

ANNUAL REVIEW OF BIOCHEMISTRY

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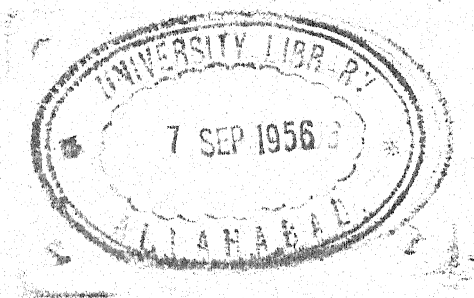
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PREFACE

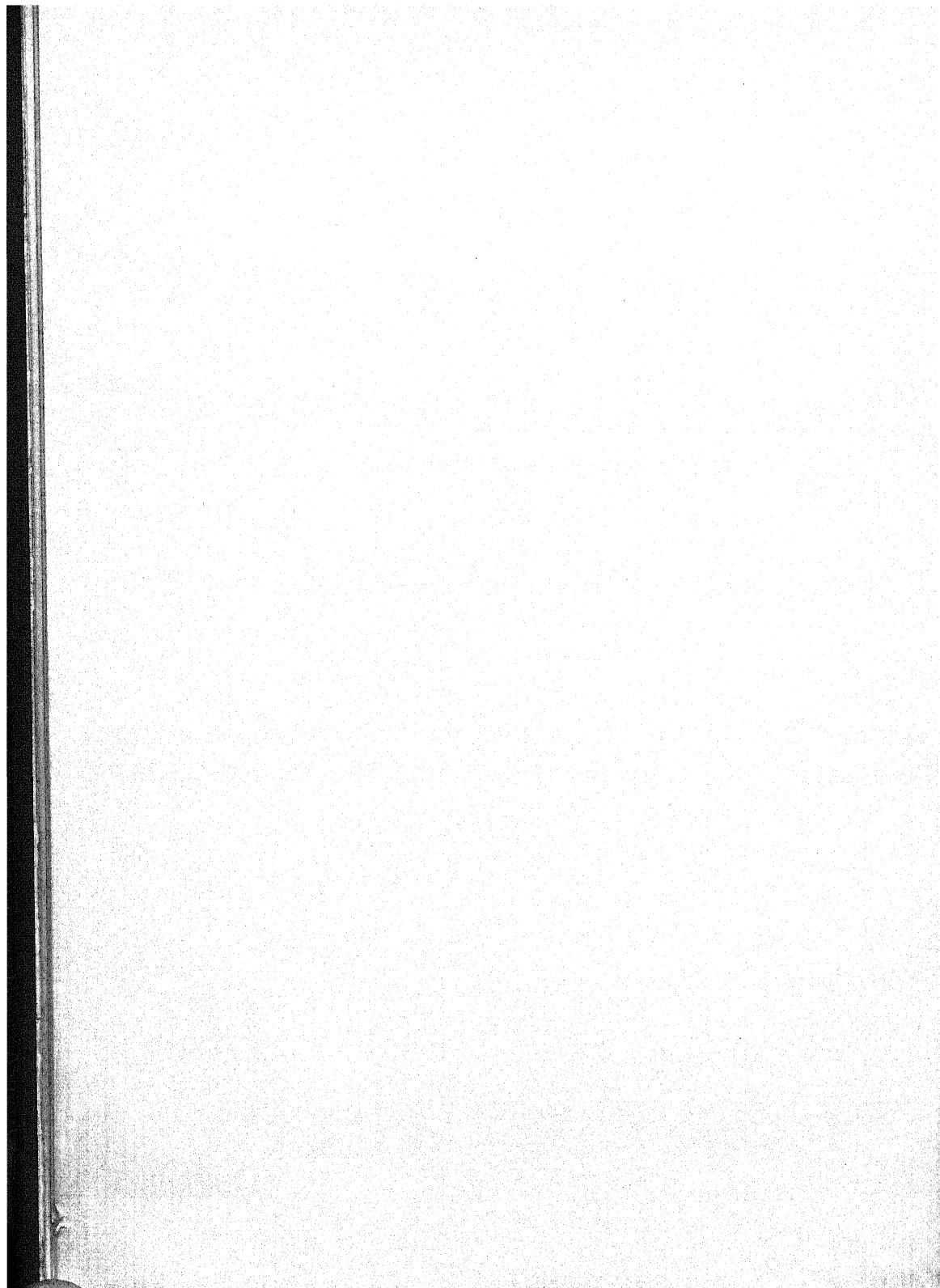
In the year that has elapsed since publication of the fourth volume of the *Review* there have been no changes in editorial or publication policy. In consequence, the need of a preface to the present volume might well be questioned were it not for the fact that such introductory paragraphs permit us to express the sense of appreciation that we feel to those whose labors and cordial suggestions are responsible for the volume.

If we may be permitted to speak for those who use the *Review*, more than a word of thanks must be conveyed to the contributors. Almost every field of biochemistry continues to receive extensive inquiry and the number of papers calling for careful appraisal steadily increases. Selection of the papers from which a review is to be woven, judicious weighing of the evidence presented, and synthesis of the material into a readable survey is a most difficult task. Perhaps the most unhappy feature is the necessity of leaving unmentioned a considerable number of excellent papers which temporarily must be placed aside because of the exigencies of space or because they pertain to subjects beyond the limits of the survey. The co-operation of the contributors has been so cordial and their attention to the exacting duties of a reviewer so complete that we wish most sincerely to endorse the sentiments of gratitude expressed by subscribers.

We continue to be indebted to the readers of the *Review* who, in increasing numbers, assist us with suggestions with respect to authorship, subjects appropriate for survey, and other matters which bear upon the quality and utility of the *Reviews*.

May we also express our gratitude to those who have been good enough to provide contributors with reprints of their published papers. In many instances, especially in the case of reports appearing in the less accessible journals, these reprints have been of the greatest assistance.

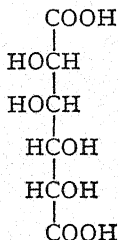
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ERRATA

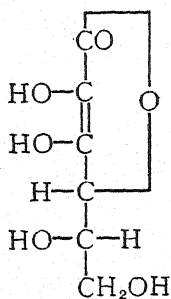
The following errors have been brought to the attention of the editor :

- Volume I, page 413: *for* Thompson, *read* Thomson.
 Page 426, line 11: *for* Cornell, *read* Corneli.
 Page 427, line 5: *for* Cornell, *read* Corneli.
- Volume II, page 38: Delete subscript 2 from the beta carbon atom in the graphic formula.
 Page 44, first formula from left should read :

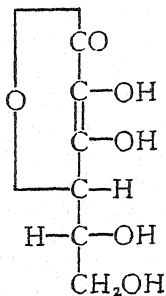


- Page 59, seventh line from bottom: *for* 2,3,6-glucopyranose, *read* 2,3,6-trimethyl-glucopyranose.
 Page 60, line 10: *for* 2,3,6-tetramethylglucose, *read* 2,3,6-trimethyl-glucose.
 Page 74, tenth line from bottom: *for* prolin: gelatin, 0.9, *read* proline: gelatin, 9.0.
 Page 101, twelfth line from bottom: *for* dl-methionine, *read* dl-Methionine.
 Page 465, fifteenth line from bottom: *for* 5,5-dimethyl-cyclohex-andian-1,3, *read* 5,5-dimethyl-cyclohexanediol-1,3.
 Page 528, line 9: *for* capronic, *read* caproic.
- Volume III, page 127, line 31: *for* increase, *read* decrease.
 Page 195, line 29: *for* histidine, *read* histamine.
 Page 197, line 12: *for* Cystine, *read* Cysteine.
- Volume IV, page 2, line 11: *omii* inversely.
 Page 51, third line from bottom: *for* arginine, *read* arginase.
 Page 100, Table II, line 2:
 for Methyl alcohol...25.7 1183 1498,
 read Ethyl alcohol...25.1 1156 1464.
- Page 163, line 22: *for* *in vitro* as *in vivo*; *read* *in vivo* as *in vitro*.
 Page 286, formula XIV: Add CH₃ group.

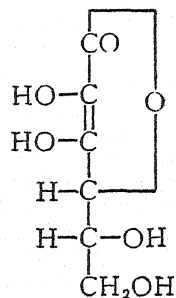
Page 349: The formulae for *l*- and *d*- and *iso*-ascorbic acid are better shown as follows:



l-Ascorbic acid
Vitamin C

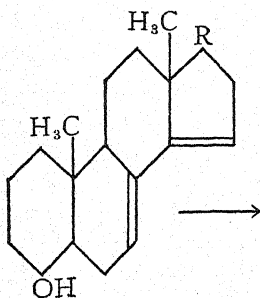


d-Ascorbic acid

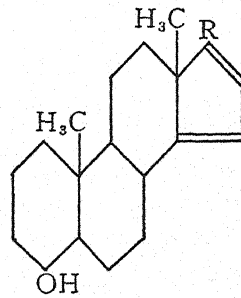
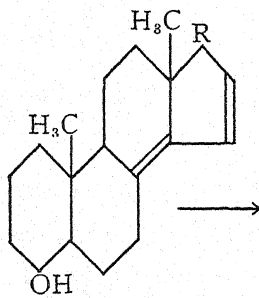


d-Erythro-3-
ketohexonic acid
"Isovitamin C"

Page 364: Rosenheim and King's suggested provisional formulae for tachysterol, intermediate product, and calciferol, should have been written:



Tachysterol



Calciferol

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BIOLOGICAL OXIDATIONS AND REDUCTIONS*

By D. E. GREEN¹ AND D. KEILIN

*Institute of Biochemistry, and Molteno Institute,
University of Cambridge, Cambridge, England*

In this review it is proposed to deal mainly with the mechanism of intracellular oxidation reactions, and with the properties of the components of cellular oxidation systems. Studies of the intermediary metabolism of animal tissue or bacterial cells will be considered here only in so far as they bear directly on the main problem.

COENZYMES

*Coenzyme of hexose monophosphoric system.*²—Warburg and his coworkers continued their investigation of the system which oxidises hexosemonophosphoric to phosphohexonic acid. This system which has been already described in the reviews of previous years is composed of a dehydrogenase (intermediary enzyme, or *Zwischenferment* of these authors), a coenzyme, hexosemonophosphoric acid, "yellow enzyme" (or flavoprotein compound), and molecular oxygen.

Warburg & Christian (1) have proceeded with the purification of the coenzyme, and from 250 liters of horse blood they have obtained 1 gm. of a highly purified product, 0.001 mg. of which can transfer 1 c.mm. of oxygen in one minute. The Q_{O_2} of the purified coenzyme is equal to 60,000, and 1 cc. of red blood corpuscles contains 0.012 mg. of this coenzyme.

Theorell (2, 3) by a cataphoretic method established that the coenzyme migrates anodically over the entire pH range, thus ruling out all possibility of a free amino group as in adenylic acid. This line of evidence was confirmed by the fact that nitrous acid does not inactivate the coenzyme. He further demonstrated the presence of two acidic groups with pK_1' equal to 1.8 to 1.9 and pK_2' equal to 6.2 to 6.3, respectively. From the fact that each acidic group involved two equivalents of hydrogen, he concluded that the coenzyme is a diphosphoric ester.

Warburg, Christian & Griesse (4, 5) culminated their research of

* Received February 19, 1936.

¹ Beit Memorial Research Fellow.

² Cf. also this volume, pp. 31, 181. (EDITOR.)

the last four years with unquestionably the most fundamental contribution that has been made to the subject of coenzymes. They proved that the coenzyme is composed of one molecule of adenine, one of β -nicotinamide, three of phosphoric acid, and two of pentose. Assuming six molecules of water of hydration, the molecular weight of the coenzyme should be 743. Molecular weight determination by the method of the depression of the freezing point of water yielded a value of 870; but considering the great uncertainty of this measurement, owing to the presence of many dissociable groups in the coenzyme molecule, the agreement is not unsatisfactory. The analysis figures agreed with the formula $C_{24}H_{28}O_{17}N_7P_3$. The experiments of Theorell (2, 3, 7) indicate that the third phosphoric acid group of the coenzyme is completely substituted and therefore does not titrate as an acid.

In presence of the dehydrogenase (*Zwischenferment*), hexose-monophosphate is oxidised to phosphohexonic acid while the coenzyme is simultaneously reduced. One molecule of hydrogen is transferred from the substrate to the coenzyme in presence of the enzyme. This reduction of the coenzyme can also be brought about by hydrosulphite ($Na_2S_2O_4$). Reduced coenzyme, formed either by enzymic reduction or by reduction with hydrosulphite, is reversibly oxidised by the yellow enzyme (flavoprotein) and the original coenzyme is regenerated. This reversible oxidation and reduction is the key to the catalytic functioning of the coenzyme. However, if the coenzyme is reduced by platinum and hydrogen in a slightly alkaline solution, one molecule of coenzyme takes up three molecules of hydrogen and forms a compound which can no longer be reoxidised by the yellow enzyme. In other words, the reduction with nascent hydrogen is an over-reduction and is not reversible, and the catalytic activity of the coenzyme is therefore destroyed.

The coenzyme has no absorption bands in the visible region of the spectrum but shows a broad band at 260 m μ in the ultraviolet, which can easily be explained on the basis of the summation of the individual absorptions of both the adenine and β -nicotinamide moieties of the molecule (the $-C=N-$ groups). On reversible reduction, this band remains unchanged and a new band at 345 m μ appears. On irreversible reduction, the band at 260 m μ fades, and the absorption that remains is due only to the adenine moiety. From this evidence, Warburg *et al.* conclude that by irreversible reduction the pyridine ring of β -nicotinamide is transformed into a piperidine ring. Piperidine

does not absorb appreciably at 260 $m\mu$ thus explaining the fading of this band on over-reduction. They have also found that if the coenzyme is previously reduced by the hexosemonophosphoric system and then treated with platinum and hydrogen, it takes up only two molecules of hydrogen instead of three in the latter reaction. These facts show that in the biological reduction of coenzyme, it is the pyridine ring only which takes up a molecule of hydrogen.

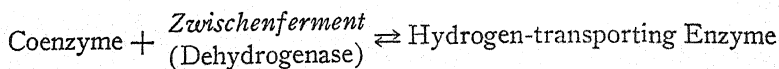
The interpretation of the spectroscopic data received confirmation from a similar study of the inner methyl betaine of nicotinic acid (trigonelline). On reversible reduction with hydrosulphite, this substance shows a new band at 345 $m\mu$, while the original absorption remains unchanged. Like the partially reduced coenzyme, reduced trigonelline is also unstable in acid solution.

The mechanism which Warburg and his coworkers propose for the reaction between oxygen and the substrate is one involving (a) the transfer of two atoms of hydrogen from the substrate to the coenzyme, (b) the transfer of two atoms of hydrogen from the reduced coenzyme to the yellow enzyme, and (c) the transfer of two atoms of hydrogen from the reduced yellow enzyme directly to oxygen with production of hydrogen peroxide. The dehydrogenase is involved in step (a) only. A pyridine and an isoalloxazine ring provide the chemical pathway for the transference of hydrogen from hexosemonophosphate to oxygen.

The reversible reduction of the coenzyme either enzymatically or by hydrosulphite has been determined manometrically in the presence of bicarbonate and 5 per cent carbon dioxide. The oxidation products of sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) or of hexosemonophosphate being acids, one molecule of evolved carbon dioxide indicates the transference of one molecule of hydrogen from the substrate to the coenzyme. Having established that the hydrogen capacity of 1 mg. of pure coenzyme is 30 c.mm., the hydrogen capacity per mg. of any preparation divided by 30 gives directly the measure of purity of the preparation with respect to coenzyme. Similarly from the known absorption coefficient of pure reduced coferment at 345 $m\mu$, the concentration of coenzyme in any preparation can be determined spectrophotometrically after addition of the reducing agent.

Warburg, Christian & Griese, in their preliminary communication (4), have already advanced the view that the function of their *Zwischenferment* is that of a protein carrier for the coenzyme. This view was more recently developed by Negelein & Haas (6). By

studying the kinetics of the reduction of the coenzyme, varying in turn the concentration of coenzyme, enzyme, and substrate, they arrived at the conclusion that the enzyme acts only as a colloidal carrier and has no catalytic activity apart from the coenzyme. The enzyme and coenzyme combine reversibly to form the catalytically active complex which is the "hydrogen-transporting enzyme."



They estimate that when the concentration of coenzyme is $10^{-5} M$ half the active complex is dissociated into coenzyme and *Zwischenferment*. One molecule of active complex in presence of hexosemonophosphoric acid can hydrogenate at least 3400 molecules of coenzyme per minute. The relationship between the coenzyme and enzyme is, according to these authors, of the same nature as the relationship between the flavin and protein in yellow enzyme or even globin and heme in hemoglobin. These results are however not in agreement with those obtained by Theorell (7) who studied the migration of hexosemonophosphate, coenzyme, and dehydrogenase in an electric field. All these components were negatively charged and migrated anodically. From an analysis of the rate of migration of mixtures of the various components, he concluded that the coenzyme formed a compound with the substrate as did the enzyme. However, there was no indication of any compound formation between the enzyme and the coenzyme.

In the theory of Warburg and his coworkers, neither the formation of the compound between the enzyme and substrate nor the activation of the latter are considered.

Cozymase.⁸—In addition to the coenzyme of Warburg & Christian there have been several coenzymes described and isolated from animal and yeast cells which are required for the complete functioning of dehydrogenase systems. Outstanding in importance among these is the Euler & Myrbäck cozymase of yeast. Warburg & Christian (1) proved conclusively that their coenzyme could not replace cozymase as the activator of alcoholic fermentation. Thus it is clear that they are not identical although their chemical composition and properties, as we shall see, have much in common.

Euler, Albers & Schlenk (8) succeeded in purifying cozymase

⁸ Cf. also this volume, pp. 30, 190. (EDITOR.)

from* ACo 110,000 to ACo 350,000 to 400,000 by precipitation as the cuprous salt. On the basis of Myrbäck's & Euler's (10) determination by the method of diffusion, Euler *et al.* (8) assumed the molecular weight to be about 394, and found the analysis figures to agree with the formula $C_{12}H_{19}O_9N_4P$. This value is half that assigned to the coenzyme from red blood corpuscles. In a later note, Euler, Albers & Schlenk (11) modified their empirical formula to $C_{24}H_{35}O_{18}N_8P_2$ which corresponds to a molecular weight of 785. Hydrolysis in acid solution yielded adenine to the extent of 19.5 per cent of the total weight of coenzyme. The amide of β -nicotinic acid was also isolated as the picrolonate but to the extent of only 47 per cent of the theoretical quantity expected on the basis of the dinucleotide structure of cozymase. The essential chemical differences between the two coenzymes seem therefore to consist in cozymase having one phosphoric acid group less than the coenzyme of blood and having less than one molecule of nicotinamide per molecule of adenine contrasted to their 1:1 ratio in blood coenzyme.

Myrbäck & Örtenblad (12 to 17) published a series of papers dealing with the chemistry of cozymase. Since the ACo's of their preparations were substantially less than 200,000 units, the purity of the coenzyme preparations under study must have been less than 50 per cent. Although the chemical analyses of these preparations are still inadequate, Myrbäck & Örtenblad (13, 17) succeeded nevertheless in obtaining some very interesting information on the chemical properties of cozymase. A strongly reducing group in the cozymase molecule could not be ascribed either to the pentose or the adenine moieties. They concluded from various lines of evidence that this reducing power was due to some nitrogenous base, as yet unknown. The isolation of β -nicotinamide by Euler *et al.* was the logical development of Myrbäck's observations and of the work of Warburg *et al.* Myrbäck found further that reduction with nascent hydrogen completely inactivated cozymase and that this inactivation was correlated with a significant change in the reducing properties of cozymase. In the light of the results obtained by Warburg *et al.* it appears that over-reduction of cozymase by nascent hydrogen explains this inactivation.

Other coenzymes.—An extensive literature dealing with the interchangeability of coenzymes for various biological processes has grown

* The unit of cozymase activity of Myrbäck (9): ACo = cc. of carbon dioxide per gm. (dry weight) per hour.

up in the last few years. Since most of the investigators worked with mixtures of coenzymes, a certain number of discrepancies was inevitable.

It was found that the coenzyme of Warburg & Christian can act catalytically with the hexosemonophosphate system of yeast and blood [Warburg & Christian (18)], the lactic and malic dehydrogenases of heart [Wagner-Jauregg *et al.* (20)], the glucose dehydrogenase of liver [Euler *et al.* (19)], and the citric dehydrogenase of cucumber seeds [Wagner-Jauregg & Rauen (21)]. The cozymase of Euler & Myrbäck works with the alcohol dehydrogenase of yeast [Euler & Adler (23)], lactic dehydrogenase of heart [Banga & Szent-Györgyi (24)], malic dehydrogenase of heart [Ogston & Green (22)], glucose dehydrogenase of liver and citric dehydrogenase of cucumber seed [Andersson (26)].

The coenzyme of heart lactic-acid dehydrogenase (27) has been assumed by Banga & Szent-Györgyi (24) to be identical with cozymase on the basis of their interchangeability. This conclusion, however, may be premature in view of the description by Banga, Szent-Györgyi & Vargha (28) of chemical properties of the heart lactic-acid coenzyme which do not correspond to those of cozymase. The non-identity of the two coenzymes is maintained also by Birch & Mann (29). On the other hand, the glucose coenzyme of Harrison has been shown by Andersson (26) to be identical with cozymase.

It may be mentioned here that according to Adler & Michaelis (30) and Ogston & Green (22), lactic dehydrogenase of yeast, for the reaction with methylene blue, does not require the co-operation of either coenzyme or yellow enzyme.

It is noteworthy that according to Euler *et al.* (19) adenylypyrophosphate has no demonstrable activity as a coenzyme of oxidative processes. There have been reports from time to time of positive results obtained with this substance but these results are probably due to contamination of adenylypyrophosphate with cozymase or other coenzymes. The rôle of adenylypyrophosphate, as shown by Parnas and his school, is that of phosphate transfer exclusively.

Euler & Adler (31) and Ogston & Green (22) independently showed that cozymase was inactive with respect to the hexosemonophosphate-dehydrogenase system in contradiction to the earlier positive finding of Euler & Adler (32). The former group of workers attributed the discrepancy in their results to an impurity in their original preparations. The impurity in conjunction with cozymase

makes for catalytic activity. They concluded that this impurity⁵ is not identical with the Warburg coenzyme. Crude preparations of hexosemonophosphate dehydrogenase of yeast are rich in this impurity, which is dialysable and thermolabile. This factor is not required for the catalytic activity of cozymase in the alcohol-dehydrogenase system. Birch & Mann (29) reported a similar separation of the lactic coenzyme into two factors.

Euler & Vestin (33) and Euler & Günther (25) pointed out that thermo-inactivated cozymase was still active as the coenzyme of lactic acid formation by muscle extract in presence of hexosediphosphate, although it was completely inactive in alcoholic fermentation by yeast. They concluded that there were two functional groups in cozymase: one concerned with oxidative and fermentation processes, the other with phosphorylation and lactic acid production.

Euler & Adler (34) found that after adding adenylypyrophosphate to the hexosemonophosphate-dehydrogenase system of yeast (composed of yeast dehydrogenase, coenzyme, and yellow enzyme) both glucose and fructose could be oxidised, although in absence of the nucleotide no oxidation of unphosphorylated hexose ensues. They showed that in their preparation of the dehydrogenase there was also present a phosphorylating enzyme capable of catalysing the transference of phosphate from adenylypyrophosphate to glucose or fructose. The phosphorylated sugar thus formed is oxidised in the usual way by the dehydrogenase in presence of the coenzyme and yellow enzyme. The phosphorylating enzyme (heterophosphatase) can be separated from the dehydrogenase and its action is not dependent upon a simultaneous oxidation process. The rôles of adenylypyrophosphate and coenzyme are therefore not interchangeable. While the former acts only as a substrate for phosphorylation of glucose, the latter is concerned with the oxidation of phosphorylated hexose thus formed. It is interesting to note that in this reaction of phosphorylation, adenylypyrophosphate cannot be replaced by either muscle- or yeast-adenylic acid or creatine or even phosphocreatine.

Runnström & Michaelis (35) report synthesis of organic phosphate as a consequence of the oxidation of hexosemonophosphate by blood hemolysate (containing the dehydrogenase + coenzyme) taking place in the presence of yeast cozymase and methylene blue. With-

⁵ Note added March 31. Euler & Adler have rejected their earlier evidence and consider the impurity to be the Warburg coenzyme [*Z. physiol. Chem.*, 238, 233 (1936)].

out yeast cozymase, no appreciable synthesis takes place. Pyocyanine can replace cozymase and methylene blue. Synthesis does not occur without oxidation but the reverse takes place usually in absence of yeast cozymase.

Schäffner, Bauer & Berl (36) find that the phosphorylation of hexose by yeast phosphatase requires the presence of the hexosephosphate-dehydrogenase system.

FLAVINS⁶

Karrer, Schöpp & Benz (37) and Kuhn, Reinemund, Weygand & Ströbele (38) independently and practically at the same time announced the synthesis of 6,7-dimethyl-9-(*d*,1'-ribityl) isoalloxazine which was shown to be identical with lactoflavin in all respects. Theorell (39) purified the prosthetic group of the Warburg yellow enzyme and isolated a phosphoric compound of the composition $C_{17}H_{21}O_9N_4P$. In contrast to lactoflavin, the prosthetic flavin contains one atom of phosphorus. Kuhn & Rudy (40) phosphorylated lactoflavin with $POCl_3$ and obtained a derivative with one atom of phosphorus. This compound, however, was found not to be identical with Theorell's flavinphosphate as shown by the inability of the synthetic flavinphosphate to combine with the carrier protein of the yellow pigment. Theorell, Karrer, Schöpp & Frei (41) prepared flavinphosphate from liver. Their preparation was not analytically pure but they were able to demonstrate a partial synthesis of yellow enzyme from the flavinphosphate and the specific protein. Rudy (42) recently announced the phosphorylation of lactoflavin by intestinal phosphatase in presence of inorganic phosphate. After several hours incubation with the enzyme system, the dialysed flavin migrated anodically, i.e., in reverse direction to lactoflavin.

According to Pett (43) bottom yeast grown in presence of cyanide, cysteine, or pyridine possessed a flavin content much higher than normal. This was correlated with a decrease in the Q_0 , although the $Q_{CO_2}^{N_2}$ remained normal. A certain amount of phosphate must be present in the medium for normal development of flavin.

Ogston & Green (22) demonstrated the inactivity of lactoflavin as a hydrogen carrier for isolated dehydrogenase systems.

Yellow enzyme of Warburg & Christian (flavoprotein compound).—Theorell (44), after a long series of purifications, succeeded in crystallising the yellow enzyme, the molecular weight of which he

⁶ Cf. also this volume, pp. 33, 189, 359. (EDITOR.)

estimates at 70,000, on the basis of 0.6 per cent flavinphosphate content in the purest preparation. When a solution of yellow enzyme is dialysed against 0.02 *N* HCl for seventy-two hours, the prosthetic group is split off and diffuses into the outside fluid. The colourless inside solution is then dialysed against distilled water to remove hydrochloric acid, and after centrifugation a clear solution of the protein component of the yellow enzyme is obtained. When flavinphosphate, prepared by methyl alcohol extraction of the yellow enzyme, is mixed with the protein separated from the enzyme, they rapidly combine, resynthesising the original compound. On the other hand, the non-phosphorylated lactoflavin does not form a compound with the protein of the yellow enzyme.

Adler & Euler (45) and Ogston & Green (22) showed that yellow enzyme can function as an efficient oxygen carrier for the isolated glucose-dehydrogenase system of liver. The latter investigators and Hahn, Niemer & Freytag (46) found similar catalytic activity of the yellow enzyme with the hexosediphosphate systems of blood and of yeast. Wagner-Jauregg & Rauen (21) reported that yellow pigment is also active in the citric dehydrogenase system of plant seeds.

Meyerhof & Schulz (47) have found that in the hexosemonophosphate system composed of the dehydrogenase, coenzyme, hexosemonophosphoric acid, and the yellow enzyme, oxygen can be replaced by NO which becomes thus reduced to N_2O . It is interesting to note that the rates of reduction of NO and of oxygen are practically the same.

CYTOCHROME⁷

Theorell (48) extracted cytochrome-*c* from horse and ox heart, and after purification by dialysis, and by acetone and ammonium sulphate fractional precipitation obtained a product with 0.17 to 0.25 per cent of iron. By cataphoretic separation of impurities, the percentage of iron rose to 0.31 to 0.33. Further treatment by precipitation of the picrolonate yielded the purest preparation with an iron content of 0.34 per cent. One gram of cytochrome-*c* of this purity was isolated from 100 kg. of heart. Cytochrome-*c* was found to be strongly basic with an isoelectric point of 9.7 according to cataphoretic data. On treatment with a platinum catalyst and hydrogen, cytochrome-*c* is reduced—one atom of hydrogen being absorbed per atom of iron in agreement with the earlier results of Hill & Keilin (49)

⁷ Cf. also this volume, p. 476. (EDITOR.)

and Green (50). Zeile (51) purified cytochrome-*c* of yeast by adsorption on and elution from kaolin. The hematin content of his purest preparation was 3.5 per cent. The isoelectric point of yeast cytochrome-*c* is 8.2. Yakushiji (52) extracted cytochrome-*c* from higher plants and algae and found the extracts to have properties similar to the cytochrome-*c* solutions from yeast. Roche & Bénévent (53) redetermined the absolute absorption spectrum of cytochrome-*c* of yeast, and confirmed the results obtained by Dixon, Hill & Keilin (54). They repeated also the experiments of Keilin (55) and Zeile (56) who showed that protohematin of blood on repeated oxidations and reductions gives rise to a hematin which is very similar to that of cytochrome-*c*. The hematin-*c* thus obtained differs according to Roche & Bénévent from all the other hematin compounds by its inability to combine with native globin to form methemoglobin. This seems to show that the elements of heme which unite with globin to form hemoglobin are not identical with those which combine with other nitrogenous substances to form parahematin and hemochromogen.

Ogston & Green (22, 57) tested the ability of cytochrome-*c*, glutathione, flavin, and yellow enzyme to catalyse the reaction of eleven dehydrogenase or oxidase systems with molecular oxygen. While yellow enzyme can act as a carrier with several dehydrogenases such as hexosemonophosphoric, hexosediphosphoric, glucose, and malic, cytochrome-*c* from yeast has catalytic activity only with the succinic dehydrogenase of animal tissues and the lactic dehydrogenase of yeast. The mechanism of this catalysis involves the collaboration of two enzyme complexes: the dehydrogenase-substrate system which reduces oxidised cytochrome and the indophenol oxidase-oxygen system which oxidises reduced cytochrome.

Haas (58) calculated from spectroscopic data the percentage of the total respiration of bakers' yeast which proceeds through cytochrome. The observed rate of reduction of cytochrome was found to account for the entire respiration within the limits of experimental error. Warburg & Christian (1) similarly calculated from the turnover of the yellow enzyme that only 1/160 of the respiration of bakers' yeast can be assumed to involve the yellow enzyme. Furthermore, systems which react with yellow enzyme are insensitive to cyanide and carbon monoxide and produce one mol of hydrogen peroxide for each mol of reduced yellow enzyme that autoxidises. Most living cells, however, are sensitive to both cyanide and carbon monoxide. The fact that *in vitro* only two out of several dehydrogenases

react with cytochrome-*c* can mean only that some essential factors or components are still missing in the reconstructed system. A striking illustration of the discrepancy between *in vitro* and *in vivo* results is given by Ogston & Green in their experiments on bottom yeast. This organism contains a relatively large quantity of yellow enzyme. Yet, although equipped with dehydrogenase systems which *in vitro* can react with oxygen via yellow enzyme, its respiration is negligible in the presence of the appropriate substrates with or without added coenzyme. The addition of pyocyanine and coenzyme produces *in vivo* an enormous increase in respiration despite the fact that pyocyanine is only one-third as active as yellow enzyme *in vitro* [Green, Stickland & Tarr (59)].

GLUTATHIONE

Meldrum & Tarr (60) found that the hexosemonophosphate system of yeast or mammalian red blood corpuscles can reduce glutathione very rapidly. Hexosediphosphate and phosphohexonic acid can replace hexosemonophosphate in the yeast system but the rate of reduction of glutathione then becomes much slower. The limiting factor in the reaction of the hexosemonophosphate-glutathione system with oxygen is the speed of autoxidation of the reduced tripeptide. The experiments of Meldrum & Tarr provide a simple explanation of the difference between the two coferments which Warburg & Christian (1) isolated from red blood corpuscles. Coferment II, unlike I, does not require the addition of carrier in order to catalyse the reaction between hexosemonophosphate and oxygen. Apparently coferment II is rich in glutathione, thereby the necessity for additional carrier is obviated. Negelein in unpublished experiments quoted by Warburg & Christian (122) independently discovered the presence of glutathione in coferment II. Wagner-Jauregg & Möller (61) reported that reduced glutathione increases the rate of reduction of methylene blue by the alcohol-dehydrogenase system of yeast. They explain the effect as one of heavy metal inactivation. Kubowitz (62) prepared ferro-glutathione and demonstrated the light sensitivity of the carbon monoxide compound formed.

Ghosh & Ganguli (63) have studied the reversibility of the glutathione system.

THE RÔLE OF FUMARIC ACID IN RESPIRATION

Gözszy & Szent-Györgyi (64), already in 1934, suggested that in cellular respiration the succinic-fumaric system may act as a link

between the substances metabolised and the oxidase-cytochrome system. This hypothesis was based mainly on the observations that the respiration of minced pigeon-breast muscle suspended in phosphate buffer solution is strongly inhibited by malonic and maleic acids. More recently, Szent-Györgyi and his coworkers (65 to 71) have revised this hypothesis and have modified it considerably; it is not the succinic-fumaric, but the fumaric-oxalacetic system which makes the link between the activated molecules of substrate and the oxidase-cytochrome system.

In this scheme the substrate molecules, activated by their corresponding dehydrogenases, react with oxalacetic acid and become oxidised while oxalacetic acid becomes reduced to fumaric acid. The latter, activated by the fumaric-dehydrogenase system, is oxidised by reacting with the oxidase-cytochrome system, not directly but through the medium of an undetermined thermolabile intermediary substance.

According to Szent-Györgyi and his coworkers the following considerations can be brought forward in support of this theory:

a) The initial rate of respiration of the pigeon-breast muscle (pulp or slices) suspended in phosphate buffer solution rapidly falls off, but can be kept up for a long period by the addition of a little fumarate or oxalacetate to the medium. The addition of this substance does not increase the initial rate of respiration but only stabilises it.

b) Oxalacetic acid added to muscle pulp in the absence of oxygen undergoes rapid reduction, which, they calculate, can account for the total transfer of hydrogen in normally respiring tissue.

c) The respiration of muscle tissue is strongly inhibited by maleic acid which affects presumably only the fumaric-oxalacetic system.

d) The addition of arsenite, which has a much greater inhibitory effect on various dehydrogenases than on the dehydrogenase oxidising fumaric to oxalacetic acid, enabled them to detect the presence of oxalacetic acid in muscle tissue kept aërobically and also to reveal the existence of fumaric dehydrogenase by the Thunberg method.

e) The oxygen uptake of muscle tissue in phosphate buffer solution is strongly inhibited by malonic acid, and this inhibition is completely abolished by the addition of fumaric acid. This observation, which was in contradiction with their previous hypothesis, is in agreement with their revised views and also connects their system with the powerful succinic dehydrogenase. They assume that in an actively respiring tissue the oxalacetic acid may undergo over-reduction to succinic acid, which is very rapidly re-oxidised back to fumaric

acid by the strong succinic dehydrogenase. The function of succinic dehydrogenase is therefore merely one of maintenance of respiration. Malonic acid which poisons this dehydrogenase causes gradual and irreversible conversion of all the available fumaric and oxalacetic acid to succinic acid, thus removing all the catalytic fumaric-oxalacetic system.

f) Although the four-carbon-atom dicarboxylic acids are not regarded as substances occurring in intermediary metabolism, the enzymes dealing with these substances such as succinic and fumaric dehydrogenases and fumarase are among the most powerful enzymes of the cell. Moreover, the affinities of these enzymes for their respective substrates being great, small concentrations of these substances are sufficient for the activity of the system. Finally, two of the four-carbon-atom substances, malonic and maleic acids, are known as powerful respiratory inhibitors of muscle tissue.

For the experimental evidence supporting these considerations, the reader is referred to the original papers by Szent-Györgyi and his coworkers.

It should be mentioned here that Elliott & Schroeder (72) and Elliott, Benoy & Baker (73), in their papers on intermediary metabolism, have proposed a cycle for the oxidation of pyruvic acid in which the end product, oxalacetic acid, forms pyruvic acid again in half the original amount.

XANTHINE OXIDASE OR SCHARDINGER ENZYME⁸

Dixon & Lemberg (74) demonstrated that nucleosides such as inosine, inosinic acid, adenosine, and muscle adenylic acid cannot be oxidised by purified xanthine oxidase without previous hydrolysis. The oxidation of these substances by milk or by crude preparations of the oxidase is due to the presence of other enzymes, nucleosidases, which first liberate free hypoxanthine from them, and it is hypoxanthine which is oxidised by the xanthine oxidase. The splitting of inosine, for instance, is due to the presence of a nucleosidase which is extremely specific towards the purine part of the molecule, being unable to hydrolyse even such a similar substance as xanthosine.

Booth (75) reopened the question of the identity of the xanthine

⁸ Cf. also this volume, p. 35. (EDITOR.)

oxidase and the Schardinger enzyme. He showed that the earlier so-called separations of the two enzymes by the method of selective adsorption was improperly controlled and did not constitute any evidence for two different enzymes. Eight other lines of evidence supplied convincing proof for the identity of the two enzymes. Booth described also what he called a mixed dismutation between salicylaldehyde and uric acid with production of hypoxanthine and salicylic acid. This reaction occurs only in the presence of xanthine oxidase. From the fact that no carrier was required to catalyse this reaction, Booth concluded that the xanthine oxidase must be able to oxidise both aldehyde and purine. If there were two enzymes concerned an intermediary carrier would have been required.

Dixon & Keilin (76) have found that xanthine oxidase is inactivated on incubation with cyanide. This inactivation, however, differs in several respects from the type usually found with other oxidation catalysts. The usual type of inhibition, which affects the oxygen uptake only, is produced instantaneously, is readily reversible, and is only partial, a definite percentage inhibition corresponding with each cyanide concentration. In this case, on the other hand, the inhibition is produced by a relatively slow reaction, is irreversible, is complete at all cyanide concentrations, and affects both the anaërobic and aërobic oxidations. Aërobically the enzyme is protected against cyanide by the presence of purines, so that cyanide added during the oxidation of hypoxanthine produces no inhibition. Anaërobically, uric acid, adenine, guanine, etc., also protect the enzyme against cyanide while hypoxanthine protects the enzyme only in the presence of hydrogen acceptor. No inhibition is produced by carbon monoxide, hydrogen sulphide, pyrophosphate, or azide even on incubation with the enzyme. The fact that the rates of this peculiar inhibition by potassium cyanide are the same for the oxidation of purines and of aldehydes forms an additional strong evidence in support of the identity of xanthine oxidase and Schardinger enzyme.

Bigwood and his coworkers (77 to 81) have attempted to show that cytochrome-*c* extracted from yeast can act as a carrier between the Schardinger-enzyme system and indophenol oxidase which they consider to be present in milk. The reduction of cytochrome-*c*, however, can be ascribed to other factors present in their enzyme preparation, and the positive but very slow indophenol reaction described by these authors is more likely due to the metal impurities present in their enzyme preparation than to a real oxidase.

Reichel & Köhle (82, 83) have studied the aldehyde dehydrogenase of liver which can either dismute or oxidise aldehydes. The two processes are competitive. Addition of oxidation catalysts such as methylene blue, quinone, and indophenol oxidase favour the oxidation and depress the dismutation of aldehyde. Potassium cyanide and hydrogen sulphide inhibit the oxidation but not the dismutation. Reichel (84) considers that lactoflavin is responsible for the dismutation of aldehyde which Wieland observed with the milk oxidase. Reichel & Nief (85) have prepared also an active citric dehydrogenase from liver.

OXIDATION OF AMINO ACIDS⁹

Krebs (86) continued his studies on the deamination of amino acids promoted by slices of tissue and by the soluble enzyme already described in his previous papers. Both slices and extracts of some tissues, such as kidney, oxidise the amino acids aëroically to the corresponding keto acids. The molecular ratio, $O_2:NH_3:keto\ acid$, is 1:2:2. According to Krebs there are in cells of various organs, but especially in kidney, two enzyme systems catalysing the oxidative deamination of amino acids: (a) A system which can be separated from cells and which is not inhibited by potassium cyanide, octyl alcohol, or drying. This system, named by Krebs "*d*-amino acid deaminase," is capable of oxidising only the foreign optical isomers of the amino acids, especially monocarboxylic monoamino acids. (b) A system which cannot be extracted from cells and which like the respiration of cells is inhibited by potassium cyanide and by octyl alcohol and destroyed by drying. This system, called by Krebs "*l*-amino acid deaminase," oxidises the natural amino acids. Krebs assumes that these two systems may have certain components in common and that the soluble enzyme of *d*-amino acid deaminase, for instance, may be a component of the *l*-amino acid deaminase system. Similar results have been obtained by Bernheim & Bernheim (87) who showed, moreover, that the majority of amino acids in presence of kidney-enzyme preparation reduce methylene blue, but that the rate of the reduction varies considerably with the amino acids and is independent of the corresponding rate of oxygen uptake. This was ultimately confirmed by Krebs, who showed that the rate of anaërobic deamina-

⁹ Cf. also this volume, pp. 36, 247. (EDITOR.)

tion of the amino acids by methylene blue is very much lower than that of oxidative deamination.

Bernheim (88) studied also the oxidation of *d*- and *l*-tyrosine by kidney and liver suspensions and kidney-enzyme preparation. Kidney suspensions and purified kidney-enzyme preparation oxidise only *d*-tyrosine. With the purified enzyme one atom of oxygen is taken up and one molecule of ammonia is liberated, this reaction not being inhibited by cyanide. Crude kidney and liver suspensions oxidise *d*-tyrosine further and four atoms of oxygen are taken up. Liver suspension, however, oxidises both *d*- and *l*-tyrosine. Bernheim, Bernheim & Webster (89) have found that *B. proteus* oxidises, decarboxylates, and deaminates some of the natural amino acids.

Keilin & Hartree (90) made a comparative study of the three oxidases of animal cells, viz., uricase, amino acid oxidase, and xanthine oxidase. They found that uricase is highly specific and catalyses only the oxidation of uric acid to allantoin. None of the mono-, di-, or trimethylated or ethylated derivatives of uric acid are oxidised by the enzyme. These derivatives, however, inhibit the oxidation of uric acid, showing that they react with the same active grouping of the enzyme molecule as uric acid itself. The oxidation of uric acid, catalysed by this enzyme, takes place only aërobically, and for each molecule of uric acid oxidised, one molecule of oxygen is reduced to hydrogen peroxide. The hydrogen peroxide is either decomposed by catalase, liberating oxygen, or utilised in the oxidation of another molecule of uric acid. It can be also utilised in promoting the coupled or secondary oxidation of "Nadi" reagent to indophenol. This indophenol reaction, however, contrary to the views expressed by Harrison (91), has no bearing at all on the problem of the indophenol oxidase.

Amino acid oxidase or *d*-amino acid deaminase of Krebs catalyses the oxidative deamination of non-physiological optically active α -amino acids to the corresponding keto acids. This enzyme catalyses also the oxidation of N-monomethylalanine [$\text{CH}_3\text{CHNH}(\text{CH}_3)\text{COOH}$] to the corresponding keto acid with the liberation of a molecule of methylamine. It does not catalyse, however, the oxidation of N-dimethylalanine, α -methylalanine or N-monomethyltyrosine. For each molecule of amino acid oxidised, one molecule of oxygen is reduced to hydrogen peroxide. Comparing uricase and amino acid oxidase with xanthine oxidase, it can be seen that they all behave as true dehydrogenases activating their corresponding substrates. The activated

substrate in the case of xanthine oxidase can react equally well with methylene blue or oxygen, while in the case of amino acid oxidase, it reacts with methylene blue fifty times more slowly than with oxygen, and in the case of uricase reacts only with oxygen. Cyanide in very low concentrations produces instantaneous, strong, but reversible inhibition of uricase, strong irreversible inhibition of xanthine oxidase (when incubated with the enzyme in absence of substrate) and no inhibition of amino acid oxidase. Hydrogen sulphide has no effect on uricase and xanthine oxidase, but produces strong irreversible inhibitions of amino acid oxidase, when incubated with the enzyme in presence of oxygen. Carbon monoxide, on the other hand, has no effect on any of these enzymes.

COUPLED OXIDATION

Keilin & Hartree (92) found that the addition of alcohol to mixtures of uricase and uric acid, or to amino acid oxidase and amino acids, doubles the oxygen uptake by these primary oxidation systems. The addition of alcohol to xanthine oxidase plus hypoxanthine or aldehyde has no effect on the oxygen uptake of these systems. If, however, in addition to alcohol, a little purified catalase preparation is added to the xanthine-oxidase system, the oxygen uptake becomes more than double that of the primary oxidation system. In all these cases alcohol undergoes a secondary or coupled oxidation to aldehyde by the hydrogen peroxide formed in the primary reaction. While catalase preparations mixed with ordinary hydrogen peroxide have no effect on alcohol, the same preparations added to other peroxides catalyse the oxidation of alcohol to aldehyde. The common property of peroxides, other than hydrogen peroxide, is the gradual liberation of hydrogen peroxide in what may be considered as a nascent state. The coupled oxidation of alcohol to aldehyde requires therefore two factors: a peroxide in nascent state which is being constantly formed in the primary oxidation reaction catalysed by the three oxidases; and a factor found in uricase, amino acid oxidase, and catalase preparations, but not in xanthine-oxidase preparations. All the evidence points to the factor being identical with the enzyme catalase. In a system composed of xanthine oxidase, aldehyde, alcohol, and catalase, aldehyde is oxidised to acid and hydrogen peroxide is formed; the latter, in turn, oxidises alcohol to aldehyde which can then be oxidised by the primary enzyme system. This type of oxidation system is described as cyclic oxidation.

CATALASE¹⁰

Stern (93) recorded that hemin, obtained from catalase preparations, when treated with hydriodic and glacial acetic acids gives a porphyrin which has the same absorption spectrum as the pure mesoporphyrin IX. The dimethyl ester of this porphyrin, when mixed with an equal amount of synthetic mesoporphyrin IX (m.p. 212°), caused no appreciable depression of its melting point. This shows that the hematin group of the catalase preparation is a derivative of aetioporphyrin III and has therefore the same arrangement of the side chains as is found in the natural blood pigment or protohematin IX.

On treating strong catalase preparation with an excess of ethyl hydroperoxide, Stern (94) finds a change in the absorption spectrum due to the formation of a compound between the hematin of the preparation and ethyl hydroperoxide. It may be mentioned in this respect that according to Keilin & Hartree (95) methemoglobin combines both with hydrogen peroxide and ethyl hydroperoxide forming peroxide-methemoglobin compounds, the formation of which requires one molecule of peroxide per atom of iron of methemoglobin. These peroxide-methemoglobin compounds are unstable and during their decomposition hydrogen peroxide is not split off into molecular oxygen and water, but is decomposed peroxidatically and both peroxides seem to be utilised in a true oxidation reaction such as is promoted by peroxidase.

Blaschko (96) investigated the effect of various inhibitors on catalase and found that the inhibitions produced by sodium azide, hydroxylamine, phenylhydrazine, monomethyl peroxide, and some other compounds are perfectly reversible. Agner (97) claimed to have separated catalase by dialysis against acid solution into two fractions, the prosthetic group and the protein, each alone being inactive but regaining activity on mixing.

MISCELLANEOUS

Mann & Saunders (98) studied the oxidation of aniline by peroxidase and hydrogen peroxide. They isolated and identified various substances formed, such as 2,5-dianilino-quinone-imide-anil, phenylphenosafranine, induline, and ungreenable aniline black. A scheme suggesting the mechanism of the reaction is given, in which phenyl-

¹⁰ Cf. also this volume, p. 35. (EDITOR.)

hydroxylamine is the primary product of oxidation and 4-amino diphenylamine is an important precursor of the end products. Fenton's reagent yields entirely different oxidation products.

Stickland (99, 100) considered the detailed chemistry of the linked oxidation reactions between amino acids in presence of *Cl. sporogenes*. When alanine is oxidised and proline or glycine is simultaneously reduced, alanine gives rise to one molecule each of acetic acid, carbon dioxide, and ammonia. The oxidation probably occurs in two stages, ammonium pyruvate being the intermediary. Glycine is reduced at the expense of alanine to acetic acid and ammonia. Proline in presence of alanine is converted to δ -amino-*n*-valeric acid.

Jowett & Quastel (101 to 103) continued their studies on the oxidation of fatty acids by tissue slices. They measured the rates of oxidation of butyric, crotonic, and *dl*- β -hydroxybutyric acid to acetoacetic acid with different substrate concentrations and in the presence of inhibitors. Acids of four, six, and eight carbon atoms produced acetoacetic acid most rapidly; the higher fatty acids and acetic acid were not easily oxidised. Odd-numbered fatty acids produced a greater increase in the oxygen uptake than the even numbered. This fact suggested that oxidation of the odd-numbered fatty acids was more complete.

Edson (104) also studied extensively the oxidation of fatty acids by tissue slices.

OXIDATION-REDUCTION POTENTIALS

Greville & Stern (105) obtained no evidence that 3,4-dinitrophenol forms a reversible oxidation-reduction system. Krah1 & Clowes (106) concluded that the principal effect of 4,6-dinitro-*o*-cresol on oxidation precedes and is not directly concerned with the activation of oxygen. Alwall (107) reviewed the literature dealing with the action of dinitrophenols and studied in great detail the effect of these compounds on respiration.

Laki (67) measured the potentials of the succinic-maleic enzyme system. Lehmann (108) and Wurmser & Filitti (109) demonstrated the reversibility of the yeast-alcohol dehydrogenase system. Filitti (110) gave the complete account of her work on the potentials of the xanthine-oxidase system and answered previous criticisms. Itoh (111) subjected the catalase-hydrogen-peroxide system to potentiometric analysis and obtained evidence for the formation of active hydrogen peroxide.

Elliott & Baker (112) studied the effect of oxidation-reduction-indicator dyes on the metabolism of tumour and normal tissues.

Stern (113) measured the potentials of toxoflavin, and Stare (114) did the same with hepatoflavin.

Stiehler & Huffman (115, 116) have determined the ΔF and ΔH values for biologically important purines.

Van Heyningen (117) finds considerable cyanide inhibition of tissue respiration in presence of phosphate, provided the cyanide concentration is maintained constant in the manometer vessels.

The reader is referred to the reviews by Borsook (118), Harrison (119), Wagner-Jauregg (120), and Shibata (121), which have appeared in Volume IV of the *Ergebnisse der Enzymforschung*.

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INSTITUTE OF BIOCHEMISTRY AND MOLTENO INSTITUTE
UNIVERSITY OF CAMBRIDGE
CAMBRIDGE, ENGLAND

ENZYMES*

By J. H. QUASTEL

*Biochemical Laboratory, Cardiff City Mental Hospital,
Whitchurch, Glamorgan, Great Britain*

ACTIVATORS AND INHIBITORS OF ENZYMES

Renewed attacks have been made this year on the problem of activation of enzymes by thiol compounds, ascorbic acid, and hydrogen cyanide. It is known that papain is inhibited by ascorbic acid alone but activated by ascorbic acid- Fe^{++} (1), that cathepsin is activated by ascorbic acid, the effect being increased by the presence of Fe^{++} (2), and that arginase is activated (3) but that urease (3) and β -malt-amylase (4) are inhibited by ascorbic acid. Purr (5) now ascribes the activation of papain by ascorbic acid- Fe^{++} to the presence of contaminating -SS- compounds which are reduced to the thiol condition by the ferrous-ascorbic acid complex. The activations of papain and cathepsin (in contrast to that of arginase) are due solely to -SH compounds (produced from -SS- by activators) and Purr concludes that papain and cathepsin are, in the active state, -SH proteins. He has also found that papain may be inactivated by alloxan, methylglyoxal, hydrogen peroxide, and the xanthine oxidase-hypoxanthine-peroxidase system; the inactivated enzyme may be restored to activity by treatment with hydrogen sulphide or cysteine. Such results would seem to indicate the presence of a thiol group in papain, as already suggested by Bersin & Logemann (6), but caution must be exercised in accepting such a conclusion. It has already been found by Quastel & Wooldridge (7) that a number of bacterial dehydrogenases (e.g., formic) may be inactivated by nitrites and partially restored to activity by treatment with sodium hydrosulphite or cysteine. It is conceivable that extraneous substances may be present in the system whose oxidation and reduction may influence the activity of the catalyst. Maschmann & Helmert (8) have studied the activating action of iron compounds on the hydrolysis of gelatin, etc., by papain and have shown that the effect is dependent upon the nature of the iron linkage. The effect would seem to be due to the removal of inhibitors as well as to the formation of new -SH groups. Addition of ferrous ions (9) increases the specificity range of papain and enables the enzyme to

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hydrolyse Witte's peptone, probably by reducing $-SS-$ groups in the substrate to the thiol condition. Activation of papain cleavage of gelatin or peptone by hydrogen cyanide is held to be due to a shift of $-SS-$ to $-SH$. The fact that the presence of ferricyanide may stimulate gelatin hydrolysis by papain (10) is possibly due to the production of ferrous ions (11). If thiol groups form a vital part of the proteinase (cathepsin and papain) molecules, it would have been thought that iodoacetic acid would exercise a powerful and irreversible effect. This seems to be true (5) but Maschmann (12) states that the activity of the proteinase is stopped by a small quantity of iodoacetic acid even in presence of an excess of cysteine. Moreover if cathepsin or papain be mixed at pH 7.6 with iodoacetic acid and allowed to stand for a short interval, the enzyme can be again extracted with alcohol and the activity is regained. This observation throws doubt on the thiol nature of papain; on the other hand it is difficult to understand why cysteine does not neutralise, at least partially, the effect of iodoacetic acid. Such neutralisation by thiol compounds of the inhibiting effect of iodoacetic acid is certainly to be observed in experiments, for instance, on glucose breakdown by brain tissue (13). Grassman (14) holds that papain preparations contain a sulphur compound which is turned into an $-SH$ compound by the action of hydrogen cyanide. This natural papain activator is not glutathione but apparently a peptide containing at least cysteine and glutamic acid. Bergmann & Ross (15) have found a series of simple synthetic substrates which are hydrolysed by cyanide-activated papain. These are acylated peptides such as carbobenzoxydiglycylglycine and even hippurylamide. The problem arose as to whether the enzyme system in papain which splits the synthetic substrates in the presence of cyanide is the same as that which splits proteins. These authors found that the activation processes of papain for gelatin and for synthetic substrates were independent of each other and that there must be in natural papain two different proteolytic enzymes, a proteinase and a polypeptidase, the former of which is reversibly inactivated by oxidation and the second irreversibly inactivated. Hence the activity of the polypeptidase (so called because of the necessity for two peptide linkages in its substrates) cannot be interpreted on an $-SH$ $-SS-$ basis. The presence of a very small quantity of phenylhydrazine will prevent the hydrolysis of synthetic substrates.

Hanes (16) has made an interesting study of the reversible inhibition of β -malt-amylase by ascorbic acid and the allied substances—

reductone and dihydroxymaleic acid. He considers that the inhibition is due to the dienol grouping common to these substances and finds that the inhibition is reversed (*a*) by oxidative destruction of the dienol group and (*b*) by the presence of reducing substances, e.g., hydrogen cyanide, $\text{Na}_2\text{S}_2\text{O}_4$, cysteine, thiosalicylic and thiolacetic acids. Methylene blue and potassium ferricyanide reverse the inhibition by ascorbic acid. Addition of ferrous sulphate has but little effect on the inhibition, but traces of copper cause increased inhibition.

According to Mayr & Borger (17) addition of cyanide increases catheptic digestion of various proteins (gelatin, serum albumin, edestin, Witte's peptone); reduced glutathione has a larger effect and a mixture of cyanide and glutathione a still larger action. The effect is probably due to the increased stability of the thiol compound in presence of the cyanide.

Activation of arginase by cysteine-Fe can always be obtained, according to Weil (18), who makes the additional observation that the livers of cancerous animals tend to be low in arginase.

Yeast proteinase is highly activated by the presence of cysteine (19) and an excess of this substance brings about inactivation.

Klein & Ziese (27) show that purified arginase is inhibited by cysteine but that the crude enzyme is activated by the thiol compound. Leuthardt & Koller (28) hold that the mechanism of activation of arginase by cysteine is concerned with a protection of the enzyme from oxygen. Hellerman & Perkins (321) find that arginase is not activated by cyanide or thiol compounds such as cysteine.

In considering all results on the activation of enzymes by reducing compounds, it is important to bear in mind the effects of such compounds on the possible natural inhibitors present. It is perhaps worth recalling that the activity of urease is inhibited by benzoquinone at very low concentrations (e.g., 1 in 5×10^6)—enzyme extracts may well contain traces of quinones—and the inhibition is reversed by thiol compounds which reduce the quinone to the inactive phenol (20).

Small concentrations of glutathione and thiolactic acid (and hydrogen peroxide) scarcely influence the hydrolysis of glycerophosphate by kidney (pig) phosphatase according to Köster & Bersin (170), but Albers (171) claims that cysteine and glutathione are inhibitory to phosphatase at low concentrations.

Arsenic compounds.—According to Maschmann (21) arsenophenylglycine inhibits hydrolysis of gelatin by papain in citrate buffer, but activates it in an acetate buffer. Arsphenamine stimulates papain

activity in both buffers. Bersin & Köster (22) have examined the effects of $p\text{-NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{AsO}$, $p\text{-CH}_3\text{CO} \cdot \text{NH} \cdot \text{C}_6\text{H}_4 \cdot \text{AsO}$, and $3,4\text{-NH}_2(\text{OH}) \cdot \text{C}_6\text{H}_6 \cdot \text{AsO}$ on papain and conclude that the thiol group of this enzyme is oxidised to the $-\text{SS}-$ condition. Arsinic oxides are more inhibitory than arsinic acids to liver cathepsin (23).

Ozone.—Ozonised water irreversibly inactivates emulsin (24), the quantity of ozone required to destroy the activity of a given quantity of almond emulsin being constant at pH 5.0 (25). Tryptophane and other indole derivatives protect emulsin from ozone but tyrosine, cystine, and maleic acid do not. α -*d*-galactosidase of alfalfa and malt diastase are also sensitive to ozone.

Magnesium.—Bamann & Riedel (26) find that the alkaline phosphoesterase (optimum pH = 9.5) of pig liver and kidney is activated by 0.01 *M* to 0.05 *M* MgCl_2 , but that the acid phosphoesterase (optimum pH = 5.5), also present, is not so activated. Magnesium activates both glycerophosphatase and saccharophosphatase (29). Holmberg (30), studying the mechanism of magnesium activation of phosphatase (intestinal), has shown that magnesium has no effect on the initial velocity of hydrolysis of glycerophosphate by the purified enzyme and supports Erdtmann's view that there exists a magnesium-enzyme complex which is not inhibited by small quantities of phosphate to the same extent as the free enzyme. The phosphatase which splits phenylphosphoric acid and α - and β -glycerophosphoric acids, and which is contained in the urine of adults, is not activated by magnesium ions (0.01 *M* or 0.005 *M*) (31).

Nickel.—According to Rosenthaler (32) the asymmetric cleavage of $dl\text{-C}_6\text{H}_5\text{CH}(\text{OH})\text{CN}$ by emulsin is catalysed by nickel carbonate. (The synthetic action of emulsin is suppressed in the presence of hydroquinone.)

Manganese and cobalt.—Arginase, according to Klein & Ziese (323), exists in normal mammalian muscle in an inactive form which is rendered active by the presence of 0.005 *M* MnSO_4 . Ferrous sulphate has but little effect. Hellerman & Perkins (321) find that arginase activity is enhanced under certain conditions by Mn^{++} , Ni^{++} or Co^{++} , as well as by Fe^{++} . Activity of the enzyme may be restored and brought to a maximum by the addition of cobaltous ions after treatment with such inhibitors as quinone, ferric ion, or iodine. Moreover cobaltous ions effect a restoration of arginase activity after this has been reduced by treatment with hydrogen sulphide. The arginase molecule is visualised as a complex containing a metal which may be sepa-

rated, e.g., by oxidation, from the rest of the molecule. The effect of Co^{++} , etc., is ascribed to the property of these ions of co-ordinating with suitable molecules to form complexes. Hellerman & Perkins also make the important observation that urease in presence of Co^{++} , Ni^{++} , or Mn^{++} will bring about the hydrolysis of arginine.

Oxalate and fluoride.—"Acid" phosphatase is inactivated by oxalate, but reactivation occurs after dialysis or precipitation of the oxalate by calcium (33). There is a less easy reversibility with fluoride. Belfanti, Contardi & Ercoli (172), investigating the mechanism of inactivation of phosphatase by oxalate, find that the latter exercises only an *initial* inhibition with "alkaline" phosphatase from liver and kidney, the inhibition disappearing with time. This is due to the formation of phosphates which compete with oxalate for the enzyme; the enzyme-phosphate complex is capable of hydrolysing glycerophosphate.

Sodium fluoride (0.02 per cent) has no effect on fumarase (34), but above the concentration 0.0005 *M* it will, in common with phosphate, inhibit glucosulphatase (35).

Dyestuffs.—Many basic dyestuffs at low concentrations, it is known, inhibit dehydrogenase activities of bacteria and mammalian tissues, the effects sometimes being reversible (36). Elliott & Baker (37) find an inhibition of oxidation in mammalian tissues by 2,6-dichlorophenolindophenol (1.3×10^{-3} *M*) and observe that the inhibitory effect of dyes may be reversible. Dickens (38) has shown that phenosafranine (10^{-5} *M*) interferes with the Pasteur phenomenon. According to Huggett (39) azo dyes—chlorazol sky blue FFS and chlorazol fast pink BKS—act as anticoagulants by inhibiting the action of thrombokinase and thrombase. Acid dyes at concentrations of 1 in 2000 and 1 in 5000 will inhibit digestion by trypsin by as much as 36 per cent (40), but Farber & Wynne (41) could find no effect of dyes on pancreatic proteinase. Quastel has pointed out (42) that the effects of dyes on urease and fumarase are influenced greatly by the presence of impurities and of substances which are themselves adsorbed by the enzymes.

Radiation.—Crabtree (43) finds that irradiation (by radium) of tumour tissue at 0° to 10° causes a selective lowering of glycolysis, with relatively little effect on the respiration, this selective action on glycolysis being more pronounced than the converse effect of irradiation on respiration at 37°. According to Harker (44) irradiation does not inhibit the indophenol oxidase of heart muscle. Brief ex-

posure of crude yeast saccharase to rays 365 to 366 m μ results in activation, lengthy exposure in inhibition (45).

Effects of phlorhizin.—Lundsgaard (46) found that phlorhizin inhibits phosphorylation in minced muscle and in yeast. Dann & Quastel (47) had previously observed that phlorhizin (1×10^{-4} M) inhibits fermentation of glucose by zymine, phloretin and phloroglucinol (the phenol present in phlorhizin) also being effective. Kalckar (48) now shows that phlorhizin is not a specific inhibitor of phosphorylation, but that it inhibits (0.02 N) the conversion of triosephosphoric acid into phosphoglyceric and glycerophosphoric acids. According to de Conciliis (49) phlorhizin has a strong inhibitory action on glycolysis in the retina of *Scyllium*, the inhibition being proportional to the concentration of drug.

COENZYMES

*Cozymase.*¹—This substance is not only important for alcoholic fermentation but it will activate the dehydrogenation of hexosemonophosphate, though it is not so active as Warburg's coenzyme in this respect (50). It also activates the glucose-dehydrogenase system of liver. It acts as an activator of glycolysis in muscle extracts (51) and of aldehyde dismutation in liver extracts (322).

According to Myrbäck (52) cozymase from muscle, liver, and kidney has the same properties as that from yeast. It is a highly reducing substance, its activity having some proportionality to its reducing power. Kidney phosphatase splits off phosphoric acid from the molecule without diminishing the reducing power though it destroys its activity. Myrbäck claims the substance to be a mononucleotide with one of the hydroxyl groups of the phosphoric acid group esterified with an unknown residue. The reducing power of cozymase resides in an unknown radical attached to adenylic acid (58), the labile reducing group probably being essential for cozymase activity. Adenylic acid deaminase (from rabbit muscle) does not deaminate cozymase, or cozymase which has been inactivated by treatment with dilute alkali (59). Purification of cozymase preparations (by means of copper) produces a compound of high cozymase activity but with no effect on hexosemonophosphate dehydrogenation, although it is still active as a coenzyme for alcohol dehydrogenation (53). Moreover cozymase which has been heated so that it has lost its property as an activator of

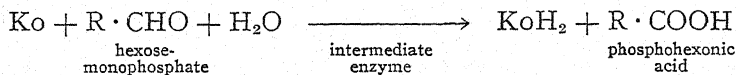
¹ Cf. also this volume, pp. 4, 190. (EDITOR.)

alcoholic fermentation will still activate lactic acid production in a muscle extract (54). Clearly, cozymase is a molecule possessing groups whose powers of activating enzymic reactions are independent or partially independent of each other. Andersson (55), who states that cozymase has no influence on xanthine oxidase but that it, in common with adenylypyrophosphate, adenylic acid, and adenosine, inhibits the Schardinger reaction, concludes that the substance has two independent reaction centres, one in an adenylic acid grouping concerned with phosphorylation and the other with oxidation-reduction.

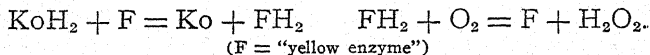
Von Euler, Albers & Schlenk (56) have analysed a highly purified preparation of cozymase whose empirical formula is given as $C_{24}H_{35}O_{18}N_8P_2$. On hydrolysis the substance yields adenine, nicotinic acid amide, and ammonia; it is probably a dinucleotide.

According to Lipmann (57) cozymase is inactivated aërobically in presence of the Schardinger enzyme.

*Coenzyme of red blood cells.*²—The coenzyme of red blood cells, important as an activator of the hexosemonophosphate-oxidation system, has now been analysed by Warburg, Christian & Griese (60). Hydrolysis of the substance yields phosphoric acid, pentose, adenine, and nicotinic acid amide, the elementary analysis agreeing with the formula $C_{21}H_{28}O_{17}N_7P_8$ (1 adenine, 1 nicotinic acid amide, 3 phosphoric acid, 2 pentose). It is clearly related to, but not identical with cozymase. The coenzyme (Ko) can be reduced by the hexosemonophosphate dehydrogenating system according to the equation:



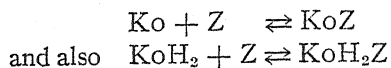
Ko is also reduced to KoH_2 by sodium hydrosulphite. The reduced form, KoH_2 , is not autoxidisable but it will reduce the "yellow enzyme" to a compound which is autoxidisable. Thus:



In this manner Ko accomplishes the activation of the hexosemonophosphate oxidation; it acts as a hydrogen acceptor, the reduced form transferring its hydrogen to a pigment ("yellow enzyme") whose reduced form is autoxidisable. Ko, in presence of platinum and hydro-

² Cf. also this volume, pp. 1, 181. (EDITOR.)

gen, in weakly alkaline solution, takes up three mols of hydrogen, the hydrogenation being irreversible. Negelein & Haas (61) find that Ko and the "intermediate enzyme" (Z) combine reversibly, thus:



KoZ is termed, or held to be the same as, the "*Wasserstoffübertragendesferment*."

The important work outlined has cleared up the relationship between "coenzyme," "intermediate enzyme," "yellow enzyme," and "hydrogen transporting enzyme." It seems to the reviewer that the stage has now been reached when the introduction of an alteration in nomenclature will be welcomed. This should make for greater clarity and help to place the enzymic system in line with other oxidising systems. The "intermediate enzyme" would seem to be similar to the Schardinger enzyme, or to glucose dehydrogenase. It appears, however, to be specific for hexosemonophosphate and Ko is presumably a specific hydrogen acceptor. The "intermediate enzyme" might be termed a hexosemonophosphate oxidase, without diminishing the significance of the former term. Moreover, there seems to be little point now in giving the combination KoZ a special name, just as there is no point in giving a special name to the reversible combination of methylene blue and succinodehydrogenase which presumably must be formed before reduction of the dye by succinate occurs.

When Ko has a concentration of $10^{-5} M$, KoZ is half dissociated (61). Phosphate ions apparently inhibit the reduction of Ko in the oxidation system. Ko does not activate fermentation (63) or alcohol dehydrogenation but it activates the glucose-dehydrogenase system (50). It also (in company with "yellow enzyme") activates citric acid and glycerophosphoric acid dehydrogenases (62) obtained from phosphate extracts of seeds and frog muscle.

Coferment T.—Although pyruvic acid activates anaërobic glycolysis in the same manner as coferment T, apparently the two are not identical (64), coenzyme preparations from beef brain not showing the presence of any pyruvic acid.

Vitamin B₁.—The coenzyme action of vitamin B₁ on lactate and pyruvate oxidation in the brain has been further studied by Peters, Rydin & Thompson (65), Rydin (66) and Kinnersley, O'Brien & Peters (67). The latter authors have shown that oxidation of vitamin B₁ does not necessarily remove its coenzyme properties. Peters

(68) finds that vitamin B₁ cannot be replaced by flavin in its effect on oxidation in the avitaminotic pigeon's brain. According to Sakai (69) avitaminosis-B in fowls decreases the hydrogen-donating power of muscle and the hexosediphosphate-dehydrogenase and lactic acid dehydrogenase activities are greatly diminished. The administration of vitamin B₁ restores normality.

Adenylpyrophosphate.—The important activating effect of adenylpyrophosphate in glycolysis and oxidations has received much attention this year. This will be discussed under a separate heading.

ENZYMES ENGAGED IN OXIDATIONS AND REDUCTIONS

"Yellow enzyme."^a—As stated earlier the "yellow enzyme" (60) acts as a hydrogen carrier, its reduced form being autoxidisable. According to Theorell (70) the "enzyme" consists of a reversible combination of a flavinmonophosphate ester with a specific protein, the compound alone being active as the hydrogen carrier. Kuhn (71) states that it is only *in vivo* that a combination of lactoflavin (vitamin B₂), or of the synthetic 6,7-dimethyl-9-*L*-araboflavin, with phosphoric acid and protein apparently takes place to form "yellow enzyme." Wagner-Jauregg and his coworkers (72) describe the accelerating action of flavoprotein on malate and hexosephosphate dehydrogenations and consider that flavoproteins may be integral components of a whole series of dehydrogenating systems. The coenzyme-intermediate enzyme-hexosemonophosphate system which reduces the "yellow enzyme" also reduces methylene blue and oxidised glutathione (73). According to Ogston & Green (74) the "yellow enzyme" shows its greatest activity towards the glucose, hexosemonophosphate, malate and hexosediphosphate dehydrogenating systems. So far as can be seen the "yellow enzyme" acts solely as a hydrogen carrier and there seems to be no point in retaining the term "enzyme" for the substance unless it can be shown that it can activate a particular substrate. Ogston & Green (74) find no basis for ascribing enzymic properties to the "yellow enzyme." Pett (75) shows that lactoflavin formation in yeast is favoured by the presence of substances (cyanide, cysteine) which poison the iron-containing catalysts. According to Elliott, Benoy & Baker (76), absence of the yellow pigment from tumours may explain their inactivity towards malate and lactate oxidations. Wagner-Jauregg & Möller (77) show that the flavin "enzyme" and

^a Cf. also this volume, pp. 8, 189, 359. (EDITOR.)

coenzyme are important factors in the dehydrogenation of alcohol by a dialysed extract from well-washed yeast. In this process cyanide and glutathione are activators, probably due to their removal of metallic inhibitors. The dehydrogenation of lactic acid by yeast (using methylene blue) is not influenced by the addition of "yellow enzyme," though that of hexosephosphate esters is affected (78).

Dehydrogenases.—Greville & Stern (79) have shown that xanthine oxidase and formic and lactic dehydrogenases, in the presence of their appropriate substrates, reduce dinitrophenol to 4-nitro-2-aminophenol. The latter two enzymes require the presence of a small quantity of a reversible redox system before reduction occurs. Xanthine oxidase and succinodehydrogenase will reduce 5,6-diketodihydroindole-2-carboxylic acid (80). Aldehyde dehydrogenase from fresh milk reduces cytochrome-*c* (81). The most important cytochrome-reducing enzyme in animal cells is succinodehydrogenase (74).

Boyland & Boyland (82) have investigated the lactic dehydrogenase activity of tumour extracts and have observed that such extracts will attack the coenzyme of this dehydrogenase so rapidly that lactic acid oxidation may not readily be seen. Such tumour extracts also contain xanthine oxidase and they will attack adenylypyrophosphate. Succinic and malic acid oxidases are absent from tumours (76) though it is possible that intermediate carriers, rather than the enzymes themselves, are missing. Lactic dehydrogenase in the tissues of young embryos is more easily damaged by γ radiation than that in adult tissues (83), the part of the reducing system injured probably being the coenzyme.

Wurmser & Fillitti (84) have found that, in presence of alcohol dehydrogenase from yeast juice and in presence of leucomethyl violet, acetone and isopropylalcohol form an equilibrium. Reichel & Köhle (85) have obtained a dry preparation of alcohol dehydrogenase by treatment of liver with acetone and acetone-ether. This preparation is more unstable than a dry preparation of aldehyde dehydrogenase made from liver by treatment with alcohol, acetone, and ether.

Ogston & Green (74) describe fresh methods for preparing lactic, succinic, α -glycerophosphoric, glucose, and malic dehydrogenases from tissues, using a steel-ball mill for crushing tissues and cells very finely. According to the same authors the principal reducing enzymes in yeast are lactic, hexosemonophosphate, hexosediphosphate, α -glycerophosphate and alcohol dehydrogenases—the succinic, malic, formic, xanthine, citric, and amino acid enzymes having but little

activity. Wagner-Jauregg & Möller (86) have found a lactic dehydrogenase preparation from *B. coli* useful as it was practically free of lyochromes; they found that its reduction of methylene blue could be accelerated by the addition of coenzyme.

According to v. Euler & Weichert (87) the succinate and malate dehydrogenase activities of germinating seeds (barley) show a rapid decrease during the first three days of growth. Succinodehydrogenase (from pig muscle) is poisoned, apparently, by 0.02 per cent selenite or tellurite, these substances being without effect on the indophenol oxidase (88).

Xanthine oxidase.⁴—Booth produces evidence showing the identity of the Schardinger enzyme and xanthine oxidase (89): competition always occurs between the relevant substrates; a reagent which inhibits the one enzyme inhibits the other; purines protect the Schardinger enzyme from destruction and no preferential destruction by heat or other agent has been found. Moreover a mixed dismutation (e.g., uric acid + aldehyde \rightarrow hypoxanthine) occurs in the absence of an added carrier.

According to Dixon & Lemberg (90) xanthine oxidase cannot oxidise any of the nucleotides or nucleosides tested; the oxidation of these substances depends on their first giving rise to free hypoxanthine by means of other enzymes.

Truszkowski & Chajkinowna (91), studying nitrogen metabolism in invertebrates, have not found xanthine oxidase in all the phyla examined though uric acid is commonly excreted. They confirmed Przylecki's rule that uricotelic organisms do not possess uricase.

*Catalase*⁵ and *peroxidase*.—According to Stern (92) the hematin group of catalase is a derivative of aetioporphyrin III and possesses a porphyrin skeleton with the same arrangement of the side chains as is found in the natural blood pigment, i.e., in protohematin IX. He considers that the high activity of catalase is due to the union of the hematin with a special protein. (Zeile in 1930 was the first to provide evidence that catalase is intimately connected with a pigment exhibiting the spectral behaviour of a complex of a hematin and a colloid component.)

If monoethyl hydrogen peroxide is added to liver catalase (pH 7.0) the enzyme spectrum vanishes and two new absorption bands become

⁴ Cf. also this volume, p. 13. (EDITOR.)

⁵ Cf. also this volume, p. 18. (EDITOR.)

visible in the green; these disappear and the original spectrum returns. The second spectrum is attributed to the formation of a compound between catalase and the substrate (92). No change in the spectrum occurs with hydrogen peroxide, presumably because of the difference between the velocity constants.

Catalases from certain species of marine plants are inactivated by oxygen (93). According to Blaschko (94) the following give reversible inhibitions of catalase: sodium azide, hydroxylamine, hydrazine, phenylhydrazine, monoethyl hydrogen peroxide, potassium perchlorate, resorcinol, and *m*- and *p*-phenylenediamine. Keilin & Hartree (95) point out that sodium azide is a strong inhibitor of catalase, and that the toxic properties of oximes found by Sevag & Maiweg are really due to hydroxylamine formed under the conditions of their experiments. Agner (96) separates the two constituents of catalase by dialysis against 0.1 *N* HCl through cellophane. The enzyme is inactivated by adsorption on glass beads but not on quartz (97). It is adsorbed by aluminium hydroxide, and the purer the enzyme the more difficult it is to remove impurities by an adsorption technique (98).

According to Ito (99) the enzyme shows two absorption bands at 406 and 266 mμ, the first corresponding to the absorption band of hemin.

Milk peroxidase, but not horseradish peroxidase, will activate persulphate as a substitute for hydrogen peroxide in the guaiacum test (100). The pH optimum of peroxidase varies with the substrate (101).

Amino acid oxidases.⁶—Krebs (102) finds that liver and kidney contain at least two different enzyme systems responsible for the oxidation of amino acids. The first is termed a *l*-amino acid deaminase which oxidises the natural (*l*-) amino acids and the second a *d*-amino acid deaminase, oxidising the foreign enantiomorphs (*d*-) of amino acids. The *d*-amino acid deaminase is not destroyed by drying, is readily soluble in water, and can be extracted from fresh and dried tissue by aqueous solutions. It is not affected by cyanide, octyl alcohol, chloroform, and toluene. It does not occur in muscle, spleen, brain, or testis. It will not attack amino acids in which the amino group is not in the α -position nor will it attack dipeptides. The *l*-amino acid deaminase is destroyed by drying the tissue, it cannot be extracted, and it is inhibited by cyanide and by octyl alcohol. It is not inhibited by arsenite, which will prevent the breakdown of α -keto acids; hence in the pres-

⁶ Cf. also this volume, pp. 15, 247. (EDITOR.)

ence of arsenite keto acids accumulate as a result of the oxidation of the amino acids.

According to Bernheim & Bernheim (104) kidney extracts preferentially attack the foreign amino acids, only *l*-proline being oxidised at an appreciable rate. Kisch (107) finds that deamination of the *d*-series of amino acids by kidney cortex occurs at a greater rate than that of the *l*-series. Weil-Malherbe & Krebs (103) show that proline, in presence of kidney slices and arsenite, is oxidised to α -ketoglutaric acid, indicating the presence of an enzyme which converts proline into glutamic acid.

Bernheim, Bernheim & Webster (105) have investigated the deaminases of resting *B. proteus vulgaris*. Apart from alanine and serine only the natural optical isomers of the amino acids were oxidised. With the exception of valine the deamination corresponded with the oxidation. Reduction of methylene blue by the amino acids in presence of the organism occurred at rates unrelated to the oxygen consumption. The oxidations were inhibited by 0.005 *M* cyanide.

According to Iyengar (106) a greater deaminase activity is exhibited by plants which are virus-infected than by the normal. This may be the reason for increased ammonia production in tobacco mosaic and spinach blight and mosaic.

Deamination of amino acids may give rise to uric acid in kidney (avian) sections [Schuler & Reindel (108)] but uric acid is not formed in liver slices. Liver slices from well-nourished animals utilise part of the ammonia for purine synthesis (108), the oxidation of purines to uric acid occurring only in the kidney. Amino acid synthesis from ammonia and ketonic acids depends on cell structure and not necessarily on respiration (109). The presence of certain amino acids (e.g., *dl*-leucine) may result, according to Edson (110), in the increased production of acetoacetic acid in rat-liver slices.

Fatty acid oxidases.—Jowett & Quastel (111) find that fatty acids with both even- and odd-numbered carbon chains are oxidised to acetoacetic acid by liver slices, a result also found by Edson (112). The yield of acetoacetic acid is considerably greater from the fatty acids with an even number of carbon atoms than from those with an odd number. The enzyme oxidising β -hydroxybutyric acid is different from that oxidising the fatty acids (111), but probably a common enzyme is involved in the oxidation of butyric and crotonic acids. The oxidation of fatty acids is inhibited specifically by low concentrations (0.001 *M*) of benzoic acid. With all fatty acids higher than valeric

acid oxidation results in the formation of an unknown fixed acid whose production is greater with the even-numbered fatty acids than with the odd-numbered fatty acids. Propionic acid does not give rise to acetoacetic acid and its oxidation is accompanied by disappearance of fixed acid. The suggestion is made that a common enzyme is involved in the initial oxidation of fatty acids. Acetoacetic acid is broken down by kidney, this decomposition being inhibited by the presence of malonate. This accounts for acetoacetic acid accumulation in tissues when malonate is added (111). Mazza (113) has investigated the oxidative action of liver on certain phenyl aliphatic acids; whereas the $\alpha:\beta$ unsaturated acid is attacked, the $\beta:\gamma$ unsaturated acid is not. Liver extracts oxidise, according to Mazza (114), palmitic or stearic acid, but not the lower fatty acids. Ghiron (115) finds that an aqueous extract of dried liver yields an enzyme oxidising fatty acids. Quastel & Wheatley (116) have shown that minced liver will not appreciably oxidise lower fatty acids.

Tyrosinase, etc.—Florence, Enselme & Pozzi (117), who have measured the changes in absorption spectra during the action of tyrosinase on tyrosine, tyramine, and adrenaline, have found no action of tyrosinase on thyroxine or di-iodotyrosine. Graubard & Nelson (118) have made an attack on the problem of tyrosinase action (*p*-cresol and catechol oxidations), concluding that tyrosinase consists of one enzyme, after a failure to separate it into constituents by dialysis, alcohol precipitations, and by adsorption and elution methods. They confirm in a number of ways the previous work of Pugh (119).

Samisch (120) has made an investigation of phenolase activity and shows that the oxidase activity is inhibited by halides of neutral salts, the inhibition being inversely proportional to the atomic number.

Fleury & Campora (121) have purified laccase but are uncertain whether manganese is a constituent of the enzyme, or whether its presence is essential for activity.

Ascorbic acid oxidation.—The finding that ascorbic acid oxidation is inhibited by tissues [Quastel & Wheatley (122), de Caro & Giani (123), Mawson (124)] has been the subject of investigation by Heard & Welch (125) who find that the perfusate of adrenal glands contains an agent diminishing the rate of autoxidation of ascorbic acid and tending to maintain it in the reduced condition. Kellie & Zilva state (126) that ascorbic acid is stable in metal-free water and that tissue extracts inhibit the oxidation of ascorbic acid in water to which iron or copper has been added. McHenry & Graham (127) maintain that

vegetable tissues do not contain a mechanism preventing aërobic oxidation of ascorbic acid. Van Eekelen (128) states that vegetables contain an enzyme which brings about partial oxidation of ascorbic acid. Tauber, Kleiner & Mishkind (129) describe an enzyme, "ascorbic acid oxidase," from the pericarp of Hubbard squash which oxidises ascorbic acid but not phenols, glutathione, cysteine, or adrenaline. It is inactivated by trypsin. They use the enzyme as a biological means of estimating ascorbic acid (130). Apparently glutathione has a retarding action on the oxidation of ascorbic acid (131). Enzyme extracts which protect ascorbic acid against oxidation accelerate destruction of reductone and dihydroxymaleic acid (16).

Fumaric acid oxidation.—According to Banga (132) there exists a "dehydrase" in minced pigeon muscle, which, in presence of a coenzyme, oxidises fumaric acid to oxalacetic acid. This reaction is held to form part of a catalytic system governing the respiration of tissues.

Oxidative enzymes in maceration juice.—Meyerhof & Schultz (133) show that oxidative enzymes (in maceration juice) bring about the reduction of NO to N_2O , the nitric oxide acting apparently directly on the oxidising agent.

PROTEOLYTIC ENZYMES

Peptidases.—Yeast peptidases readily hydrolyse such glutamine peptides as *d*-glutaminylglycine, *d*-glutaminyl-*d*-glutamic acid, and *d*-glutaminylglycylglycine (134) and the peptides show the labile characters of *d*-glutamine. The results offer evidence for the view that the ammonia produced during digestion of proteins by peptidases may arise from spontaneous breakdown of *d*-glutamine, or glutamine peptides. A crystalline globulin has been prepared from pancreas by Anson (135) which will split the amide linkage of chloroacetyltyrosine and recrystallisation does not alter its activity.

Bergmann and his colleagues (136), in an investigation on the specificity of dipeptidases, conclude that if a dipeptide contains the antipode of a natural amino acid, $NH_2 \cdot CHR \cdot COOH$, and R is CH_3 , or larger, then steric hindrance of dipeptidase action may occur; if $R = C_4H_9$ or a longer side chain, complete inhibition of hydrolysis may result. The primary cause of optical selectivity of dipeptidase lies in the fact that the enzyme combines with more than two points of the substrate, i.e., the $-COOH$, $-NH_2$ and peptide-bond H. Aminopeptidase and carboxypeptidase also need, in addition to the peptide bond, a free amino group or a free carboxyl group (137). Papain does not

split a peptide linkage next to a free alpha-amino group and does not need a free alpha carboxyl group; it does need another peptide linkage in addition to the one which is hydrolysed. Hydrolysis with papain is not restricted to the ends of the peptide chain. Each peptide linkage does not show the same behaviour to papain; the splitting rate is influenced by the nature of the side chains of the amino acid residues involved. When a peptide hydrogen atom is substituted by a methyl group the bond becomes more resistant (138).

Grassman *et al.* (139) have investigated the inhibitive action of amino acids on the hydrolysis of dipeptides by yeast and kidney dipeptidase. The affinity of the dipeptide to the enzyme is largely determined by that part of the molecule carrying the free amino group, the carboxyl portion being of less importance. The affinity increases with the length of the carbon chain. Synthesis of peptides can be demonstrated with the aid of intestinal peptidase and papain cyanide (140). Mould enzymes (155) will split benzoyl-*dl*-leucylglycine and hippuric acid. *Aspergillus parasiticus* (324) contains an aminopolypeptidase, which requires a free amino group as a point of attachment to the enzyme, and which hydrolyses the peptide linkage adjacent to this free amino group. Dipeptides are not attacked unless the carboxyl group is removed. The mould enzymes also attack peptides in which the free amino group is methylated or replaced with a chlorine atom. Crude mould peptidase splits triglycine and diglycine.

Chymotrypsinogen.—Chymotrypsinogen, a new crystalline protein from acid extracts of pancreas, may, according to Kunitz & Northrop (141), be converted into an active proteolytic enzyme, chymotrypsin, by the addition of a minute quantity of trypsin. The activation cannot be accomplished by enterokinase, pepsin, inactive trypsin, or calcium chloride. The active enzyme hydrolyses casein more slowly than trypsin, but carries out the hydrolysis further and attacks different linkages.

Trypsin and pepsin.[†]—Gorter *et al.* (142, 143) have studied the behaviour of pepsin and trypsin by the surface-film method. They reach the conclusion that the behaviour of pepsin may be explained by assuming that the enzyme is formed by a combination of a protein with a fairly strong multivalent acid neutralising most of the amino groups of the protein molecule and that it is reasonable to suppose that trypsin is a complex protein consisting of a combination of a protein and a

[†] Cf. also this volume, p. 134. (EDITOR.)

multivalent base. When myosin is hydrolysed by trypsin there is an initial formation of "spreading" substances. The limitations of the surface-film method of investigation of enzymes are discussed by Schulman & Hughes (147), who have found that purified enzyme will hydrolyse protein monolayers as they will in the bulk phase.

There occurs disaggregation rather than a proteolysis of wheat proteins by enzymes present in flour (144) which thereby have a solvent effect. The action is most marked at pH 8.5.

The effects of halogen salts on the clotting of milk by trypsin have been studied by Clifford (145). The results agree with the view of Kleiner & Tauber (146) that clotting of milk is due to a depression of proteolytic action, allowing the casein stage to show, since Clifford has shown that halogen salts of lithium, sodium, and potassium depress the proteolytic action of trypsin. The explanation is not complete, however, because fluorides, which are the greatest depressors of proteolytic activity, are least active in promoting milk coagulation. According to Mellanby (148) oxalated plasma may be coagulated by trypsin, so long as the latter contains sufficient calcium to turn prothrombase into thrombase.

Carbobenzoxylglycylglutamylglycine ethyl ester is split by pancreatic and crystalline trypsin into carbobenzoxylglycine and glutamylglycine ethyl ester (149), the suggestion being that the extra carboxyl group of the glutamic acid group enables the enzyme to combine with the molecule. In proteins both carboxyl groups of this type and terminal amino groups of lysine play a similar rôle. Trypsin (without the aid of kinase) destroys oxytocin (150). It also inactivates tobacco virus (151) though the inactivating action here may not be due to proteolysis. The tryptic digestion of globulins may be followed by dilatometric methods (152), the dilatometric depression being proportional to the release of amino nitrogen. According to Mardashev (153) frog pancreas contains a trypsin which does not differ from trypsin of warm-blooded animals. Shibata (154) points out that although neutral diketopiperazines are not attacked by trypsin or pepsin, those with a free carboxyl group are hydrolysed by trypsin and those with a free amino group are split by pepsin. According to Cohn & White (156) raw egg white contains a factor which is moderately heat labile and which resists the action of trypsin and pepsin. The rate of formation of trypsin from trypsinogen is directly proportional to the quantity of kinase added [pH 8.0 to 9.3 (157)], but according to Bates & Koch (158) there is no evidence that trypsin is a trypsino-

gen-enterokinase complex. Enterokinase acts as an enzyme in activating trypsinogen. The activation of proteolytic enzymogens by exposure to acid conditions has been studied by Ege & Obel (160). The cleavage of clupean by trypsin preparations has been investigated (159) and the contention of Waldschmidt-Leitz & Akabori that crystalline trypsin has a greater range of activity than protease purified by alumina adsorption could not be corroborated. Trypsin cannot attack (in contrast to erepsin) *l*-pseudoleucyl-*l*-tyrosine (161). Erepsin cannot attack *d*-pseudoleucyl-*l*-tyrosine. Philpot (162) has continued his investigation of pepsin by means of the ultracentrifuge and has shown that the alkali inactivated enzyme is homogeneous, differing from alkali denatured proteins which are not homogeneous. A change occurs, however, in the molecule which makes it more easily "denatured" by acid. Linderstrøm-Lang and his colleagues (320) have continued their important work in enzymic histochemistry, studying in particular pepsin, peptidase, and esterase distribution in gastric mucosa, and the distribution of enzymes in the stomach as a function of its histologic structure.

Pancreatic proteinase.—Farber & Wynne (163) show that the rate of protein disappearance in presence of pancreatic proteinase is greater than the rate of amino nitrogen accumulation, the latter not being a satisfactory index of the enzyme activity. Their results support Northrop's conclusion that the enzyme is active only on protein anions. They find (41) that the enzyme is inhibited by mono- and disaccharides at 0.5 *M*, this possibly being due to a disturbance of the effective water concentration. Aspartic and glutamic acid in contrast to other amino acids have accelerating actions. The enzyme is activated by cyanide (not due to removal of basic metals), calcium chloride, and ferro- and ferri-cyanides.

Salivary proteinase.—An enzyme apparently exists (164) which causes autodigestion of saliva but not digestion of heated egg white or fibrin.

Clothes-moth (Tineola biselliella) proteinase.—According to Linderstrøm-Lang & Duspiva (165) an enzyme exists which will break down wool keratin at pH 9.3 after the keratin has been reduced. It occurs in the secretion of the middle intestine of the clothes moth as a powerful proteinase which, in contrast to animal trypsin-kinase, is little sensitive to the action of $-SH$. Digestion of sheep wool by the proteinase, using sodium thioglycollate as reducing agent, proceeds rapidly, producing equivalent quantities of amino and carboxyl groups.

Whereas the monocytes of rabbits contain only pepsin, the polymorphonuclear cells contain pepsin, cathepsin, and trypsin (166).

Pett (167) has found that the dipeptidase and protease of scutellum and embryo of wheat seeds greatly increase during the first twelve hours of germination, after which they decrease in the embryo and continue to increase in the scutellum. Melanosarcoma (from horses) has a high content of cathepsin (168). The latter enzyme may be activated by an aqueous glycerol eluate from spleen (169).

PHOSPHATASES,^s PHOSPHOESTERASES, PHOSPHORYLASES

Liver and kidney extracts contain both "acid" and "alkaline" phosphatases, the activity of the former, but not the latter, being inhibited by fluoride, oxalate, and sodium trichloroacetate (173). Bamann *et al.* (174) find that the two enzymes are best obtained from liver by autolysis, the "alkaline" phosphoesterase (optimum pH = 9.5) having a greater affinity than the "acid" enzyme (optimum pH = 5.5) for its substrates. According to Waldschmidt-Leitz & Nonnenbruch (175) only the "alkaline" phosphatase is typical for organs, the presence of the "acid" enzyme being due to the presence of blood which contains only the "acid" phosphatase. Folley & Kay (176) have examined the "alkaline" phosphomonoesterase of mammary gland, whose function, presumably, *in vivo* is the rapid synthesis of phosphoric esters, and find it to be identical in all respects (e.g., magnesium activation, pH optimum, etc.) with kidney phosphatase. An "alkaline" phosphatase (optimum pH = 9.6) can be obtained from dog faeces (177), the optimum magnesium concentration being $< 0.0003 M$. A phosphatase, inhibited by phlorhizin, exists in retina (178) and the enzyme is also to be found in an extract of prostate (179). The optimum pH of β -glycerophosphatase in the erythrocytes of normal humans and cancer patients is 5.6 to 5.8, cancer erythrocytes being more active than the normal (180). Roche & Latreille (181) have prepared phosphatase from red blood cells; after adsorbing the enzyme on kaolin and eluting it with 0.05 *N* ammonia, it gave a pH optimum of 8 for α -glycerophosphate, and of 9 for β -glycerophosphate. The phosphatase activity of blood plasma is significantly raised in the osteoblastic type of osteogenic sarcoma (182). According to Auchinachie & Emslie (183) plasma phosphatase (of fowls) may be changed in cases of insufficiency of vitamin D and calcium.

^s Cf. also this volume, pp. 197, 305. (EDITOR.)

Tanko & Robison (184) have shown that fructose-1-6-diphosphate is hydrolysed by purified bone phosphatase to equal quantities of fructose-1-phosphate and fructose-6-phosphate. Also derivatives are only found when a phosphohexokinase is present. This kinase which is present in marrow extracts is probably identical with the enzyme found by Lohmann (185) in extracts of muscle, kidney, liver, brain, and yeast; it converts fructose-6-phosphate and glucose-6-phosphate into equilibrium mixtures of keto and aldo esters. It has no effect on fructose-1-phosphate and none on fructose-1-6-diphosphate, unless phosphatase is present to remove phosphate from position 1. According to Sacchi (186) phosphatase of bones of rats decreases markedly when the animals are kept away from sunlight for a week. The phosphatase content of the long bones of rats reaches its maximum when the animal has grown to a quarter of its maximum weight (187). An increase in phosphatase content of bone and callus is observed in healing fractures in rabbits (188), a parallel increase in serum phosphatase not taking place. Wilkins & Regen (189) also observe that after fracture of adult rabbit bone the phosphatase activity of the tissue at the site of injury rises rapidly, the maximum being reached about the twenty-second day. As repair progresses the enzyme activity decreases.

A good autolysing agent for releasing phosphatase from animal organs is ethyl acetate (218). Kidney phosphatase has a molecular weight of 6,000 to 10,000 according to Albers & Albers (218).

According to Schäffner & Bauer (190) yeast phosphatase differs from animal phosphatase by a higher specificity; it attacks α - and not β -glycerophosphate. Albers & Albers (191) state that top yeast contains one phosphatase which splits both α - and β -glycerophosphates, but that bottom yeast contains the top yeast phosphatase and two other phosphatases, one specific for α -glycerophosphate and the other for the β -ester. During the course of dialysis top yeast phosphatase undergoes a sudden and irreversible inactivation; magnesium ions inhibit it. Schuchardt (192) could not confirm Schäffner & Bauer's observation that yeast contains only an α -glycerophosphatase. Using dried yeast he finds that at pH 4 the β -ester, and at pH 6 the α -ester, is more easily hydrolysed. According to Courtois (193) the optimum pH of hydrolysis of α - and β -glycerophosphates by takadiastase varies with the concentration of substrates; the optimum pH is determined by (a) stability of combination between the enzyme and the ester and (b) the initial velocity of hydrolysis. Phenyl esters are hydrolysed by animal

phosphatases at a greater rate than ethyl esters and mono-esters faster than di-esters (194). According to Hotta (195) phosphatase specificity is determined not only by the nature of the esterification of the phosphoric acid group, but by the nature of the alcohol.

Esterification of glucose, fructose, and galactose (but not mannose and xylose) will occur in presence of a glycerol extract of rat intestine in phosphate buffer (196). Rudy (197) claims that lactoflavin can be phosphorylated by an intestinal phosphatase.

Recent very important work has shown that enzymes (which help to control glycolytic processes in tissue extracts) exist which can phosphorylate a molecule by transferring to it, apparently directly, a phosphoric acid group from a molecule which is already phosphorylated. Thus, according to Parnas, Ostern & Mann (198), the synthesis of phosphocreatine from creatine at the expense of phosphoglyceric acid by muscle pulp in presence of iodoacetic acid cannot be accomplished by free phosphate ions or by any intermediate product of glycogenolysis. Schäffner *et al.* (199) state that yeast phosphatase is not a component of the phosphorylation process. Von Euler & Adler (200) suggest the name "heterophosphatase" for the enzyme which accomplishes the transfer of a labile phosphate grouping from adenylypyrophosphate to hexose. It seems to the reviewer, however, that since the function of the enzyme is to phosphorylate a molecule at the expense of an already phosphorylated molecule, free phosphate apparently not being involved, a more suitable name for the enzyme would be "phosphorylase." It seems from the work of Lohmann (201), Needham & van Heyningen (202), Ostern *et al.* (203), Mann (204), Lutwak-Mann (205), Parnas *et al.* (206), Meyerhof & Kiessling (207), and Lehmann (208), that one or more phosphorylases govern the following reactions:

- (a) $2 \text{ Phosphoglyceric acid} + \text{adenylic acid} \rightleftharpoons \text{Adenylypyrophosphoric acid} + 2 \text{ pyruvic acid}$
- (b) $\text{Adenylic acid} + 2 \text{ phosphocreatine} \rightleftharpoons \text{Adenylypyrophosphoric acid} + 2 \text{ creatine}$
- (c) $\text{Adenylypyrophosphoric acid} + \text{hexose} \rightleftharpoons \text{Hexosediphosphoric acid} + \text{adenylic acid}.$

Reaction *a* proceeds under conditions where there is little or no free phosphate, an extract being used (202) which was dialysed sixteen hours, as this treatment (201) damages or destroys the enzyme responsible for adenylypyrophosphate breakdown while not affecting the enzyme governing *a*. The important concept of phosphate donators

and acceptors has arisen, phosphocreatine and adenylypyrophosphate, and phosphoglyceric acid being phosphate donors in presence of their appropriate enzymes. Phosphopyruvic acid is probably an intermediate in reaction *a* (204, 205). The reaction between phosphopyruvic acid and adenylic acid is inhibited by sodium fluoride which does not, however, influence the formation of adenylypyrophosphate from adenylic acid and hexosediphosphate (209). Magnesium ions are important for reactions *a* and *b* (208, 210). The following reaction also takes place in heart-muscle extract (210):

Phosphoglyceric acid + adenylic acid \rightarrow Diadenosinepentaphosphoric acid. According to Neuberg (211) the addition of fresh yeast to a mixture of fructose monophosphate and adenylic acid leads to adenylypyrophosphate and hexosediphosphate formation. Further knowledge of the properties of the phosphorylases and of their relationship to phosphatases will be awaited with great interest.

According to Satô (212) a phosphoesterase splits off one molecule of orthophosphate from adenylypyrophosphate, leaving, however, an intact pyrophosphoric nucleus still attached to the ribose, while a pyrophosphatase produces an adenosine diphosphoric ester which in presence of phosphomonoesterase loses both molecules of orthophosphate. Lohmann (213) states that dialysed crab-muscle extract removes one molecule of orthophosphate from adenylypyrophosphate, dephosphorylation of the resulting adenosinediphosphate occurring after addition of magnesium ions. In the same extract arginine-phosphoric acid is hydrolysed in presence of adenosinediphosphate but not of adenylic acid. According to Schöffner *et al.* (214) purification of yeast maceration juice removes an enzyme necessary for conversion of hexosemonophosphate into the diphosphate. A phosphatase, derived from various sources, will split phospho-*l* (+) -lactic acid forming *l* (+) -lactic acid (215). Takaphosphatase hydrolyses the potassium salt of dihydroxyacetone monophosphate [or its bisulphite (216)]. An amylophosphatase from barley, which can be separated from amylases by adsorption on alumina and kaolin, splits orthophosphate from organic bound phosphoric esters in starch paste (217).

ENZYMES ENGAGED IN GLYCOLYSIS

Enolase and phosphoglyceromutase.—The former enzyme is responsible for the reaction, phosphoglyceric acid \rightleftharpoons phosphopyruvic acid, and the second for the reaction 3-phosphoglyceric acid \rightarrow 2-phosphoglyceric acid. Their presence in dialysed muscle extract is

shown by Meyerhof & Kiessling (219). The separation of the two enzymes (in dialysed yeast juice) by adsorbing the phosphoglyceromutase on animal charcoal is described by Akano (220). Muscle, kidney, and intestinal cells and especially extracts from Jensen sarcoma accomplish the change of phospho- and diphosphoglyceric acid to pyruvic acid (221). Enolase is present in fresh muscle extract, muscle pulp, or hemolysed blood (222). Germinated seeds of *Sorghum saccharatum* change glycerophosphate into pyruvic acid (223).

A zymohexase brings about the following reversible change according to Meyerhof & Lohmann (224):

Hexosediphosphoric acid \rightleftharpoons 2 dihydroxyacetone phosphoric acid, the enzyme being present in muscle, yeast, heart, brain, retina, and spleen but not (or in traces only) in liver or kidney. The velocity is determined by a unimolecular intermediate reaction (227). The equilibrium constant of the reaction is 1.8×10^{-4} at -7° and 2.2×10^{-2} at 70° . Dialysed tumour extracts contain zymohexase (225), the activity of tumour being about one-tenth that of muscle of the same animal. According to Boyland & Boyland (225) dialysed tumour extracts convert hexosediphosphate into lactic acid so long as sufficient adenylypyrophosphate is present. Scharles *et al.* (226) maintain that tumour extracts convert hexosemonophosphate into lactic acid without the aid of a dialysable coenzyme.

Glyoxalase.—Acetone-ether treatment of liver can be made to give fairly active dry preparations of glyoxalase (228). Top yeast and various bacterial preparations transform *p*-tolylglyoxal into *p*-methyl mandelic acid, the optical rotation of the latter varying according to the source of enzyme (229).

The reaction between methyl glyoxal and glutathione which, as was shown by Jowett & Quastel (230), gives rise to the substrate from which glyoxalase forms lactic acid, has been further studied by Giršavičius & Heyfetz (231). The reaction is unimolecular but apparently the thiol group is involved in some further transformation which prevents its appearance as the enzyme reaction proceeds.

Woodward (232) makes use of the coenzyme action of glutathione in glyoxalase activity for the manometric determination of glutathione. Schubert (233) has shown that both methylglyoxal and phenylglyoxal react with thiol acids, the reaction being unimolecular. Glyoxalase activity is inhibited by amino acids, chiefly histidine (234), but that antiglyoxalase action of pancreas is due to histidine is disputed by Schröder *et al.* (235, 236) who claim that this action is due to enzymic

hydrolysis of glutathione. Goddard & Schubert (237) show that iodoethyl alcohol reacts with $-SH$ groups at a slower rate than iodoacetic acid, this fact explaining the feeble activity of iodoethyl alcohol in inhibiting glycolysis.

CARBOHYDRASES

Amylases.—Hydroxyethyl starch (prepared by passing ethylene oxide into alkaline starch) is hydrolysed by α -amylase which is activated by amylokinase from green malt extract. β -amylase of ungerminated wheat is inert. As digestion of the hydroxyethyl starch proceeds the viscosity falls without a development of reducing power, indicating the possibility of an anhydride structure in the fragments produced (238). Willstätter & Rohdewald (239) use the term "isodynamic" to describe enzymes acting on the same substrate. Isodynamic amylases include saccharogenic and dextrinogenic enzymes, amylases which are distinguishable by activation or inhibition by phosphate or glycerol, and the lyo- and desmo-amylases distinguished primarily by solubility. Amylases from animal organs belong to the α - or dextrinogenic type. Polyglucide-protein complexes (glycogen- or starch-myosin) are less readily hydrolysed by amylase than the free polyglucide (240). Giri (241) has reached the conclusion that purified amylase (of sweet potato) is not a protein and shows (242) that the enzyme is activated by anions, the effect decreasing in the order F^- , Cl^- , SO_4^{2-} , NO_3^- . There is a suggestion (243) that liver amylase regularly accompanies storage or release of free glycogen. Pringsheim & Ginsburg (244) state that it is possible to get a complete hydrolysis (to maltose) of starch by pancreatic amylase without separation of free phosphate. The action of β -amylases on various starches has been studied by Samec (245) and the effects of salts on amylase by Bauer (246) who has developed an empirical equation from which the rate and temperature coefficient of hydrolysis of starch in presence of chlorine ions may be calculated. A mode of estimating amylase activity by determining residual starch is described by Caldwell & Hildebrand (247). In the saccharification of starch by takadiastase there is formed an intermediate polysaccharide, soluble in 95 per cent alcohol, besides dextrin and maltose (248). The diastatic activity of ungerminated grain is increased by rennin (249). Human seminal plasma exhibits diastatic activity (250).

Emulsin.—Glucose, but not lactose, has some protective effect on emulsin against heat inactivation. Fluorescein and congo red do not elevate the temperature of inactivation (251). The introduction of a

methyl group in the 6-hydroxy position in β -*d*-glucosides retards (but incompletely) cleavage by almond emulsin (252). β -*d*-xylosides are split by emulsin (253), which will also hydrolyse cellobionic acid [β -glucosidogluconic acid (254)]. Almond emulsin hydrolyses *o*-cresol- α -*l*-arabinoside about 15 times more rapidly than the corresponding phenol-arabinoside (255). Helferich & Schmitz-Hillebrecht (256) have also investigated the action of neutral salts on almond emulsin showing that the activation by anions (cations being ineffective) follows the lyotropic series. Different substrates, however, are not affected by the ions to the same extent. The activation is not due to peptization of the enzyme and it is possible that the differential salt activation may provide a means of distinguishing between glucosides in mixtures.

Glucosidases.—The hydrolysis of lactose or lactose ureide by an enzyme of *Aspergillus oryzae* is affected by galactose and not by glucose, the enzyme therefore not being emulsin; that of salicin is affected by glucose and not by galactose (257). Acetone-ether dried preparations of liver and kidney will hydrolyse lactose and lactose ureide (258). According to Rabaté (259) leaves of *Salix purpurea* contain an enzyme (not emulsin) hydrolysing glucosides of various phenols (a similar preparation has been called "salicase"). The fresh and dried leaves of *Digitalis lanata* and of *Digitalis purpurea* contain desmo-enzymes splitting glucosides and are called "digilanidase" and "digipurpuridase" (260). Bacterial carbohydrases have an optimum pH of 6 to 7 (excepting sucrase whose optimum pH is 4.5), being distinguished in this way from moulds where the pH optimum lies between 4 and 5 (261).

Invertase.—Very pure preparations of sucrase have been obtained by Albers & Meyer from yeast autolysates by direct adsorption on alumina- C_γ (262). The inversion of sucrose by sucrase takes place in two stages according to Horiba & Kosaki (263), in the first of which the $\frac{dx}{dt} - t$ curve is linear and in the second of which the $\frac{dx}{dt} - k_m$ curve is linear. The inversion is not influenced by the viscosity of the medium and no inversion will take place below a water concentration of 4.75 per cent at 25° (264). The enzyme is activated by short exposure to radiation 365 to 366 m μ (265). According to Saul & Nelson (266) the presence of protein protects invertase from irreversible inactivation on standing at room temperature at pH 3.0.

Waksman & Allen (269) describe an "alginase" from marine bac-

teria which hydrolyses alginic acid (a polymer of mannuronic acid) into simpler groups of mannuronic acid but not into simple units.

An "amylosynthase" (267) which is abundant in polished rice can be prepared by treating yeast extract and also seed extracts with purified papain. Grant (268) finds that synthesis of lactose by slices of mammary gland occurs in presence of glucose but not in presence of galactose.

ESTERASES AND LIPASES

Cholinesterase.—This esterase which may be estimated manometrically is present in human sera and corpuscles and in brain but not in cerebrospinal fluid (270). It is specifically inhibited by eserine or physostigmine, the inhibition also occurring *in vivo* (271). The enzyme exists in the blood of *Octopus vulgaris*, *Aplysia depilans*, *Murex* (272), and *Helix pomatio* (273). It may be purified (274) by adsorption from acid solutions on alumina and ferric hydroxides, elution being effected at first by 0.025 *N* ammonia and then as purification proceeds by 0.3 *M* phosphate at pH 8.0. The preparation has no activity towards methylbutyrate.

Liver esterases.—Baker & King (275) show that ethylbutyrase, after much purification, is quite distinct from lecithinase, sulphatase, tannase, phytase, β -glycerophosphatase, or hexosediphosphatase. The change in inhibiting power of salts of fatty acids on liver esterase coincides with the formation of micelles and colloidal aggregates and with the changing power to lower surface tension. There is an increase in inhibiting power up to sodium laurate and then a decrease for palmitate and stearate (276). According to Bamann *et al.* only one per cent of liver esterase can be extracted by 100 per cent glycerol, about 40 per cent by dilute glycerol, and the remaining enzyme may be extracted with dilute alkali. A good yield may be obtained by allowing minced liver to autolyse at 20° in presence of *N*/20 ammonia, treating with *N*/20 acetic acid, and dialysing the filtrate (277). The enzyme may be separated from phosphoesterase (278).

Tributyrynase, cholinesterase, and methylbutyrase exist in the hen's egg; they undergo no change during the first five days of incubation and then increase rapidly (279). The lungs of rachitic rats show marked differences in the content of esterase from the normal (280). The tributyrinase activity of rat brain is fifty times less than that of the liver. Organs of tumourous animals have less lipolytic power than the normal (281). The distribution of lipase and esterase activities in

the adrenal gland has been described by Glick & Biskind (282). According to Kirsh (283), the lipases of *Penicillium oxalicum* and *Aspergillus flavus* have pH optima at 5.0, the lipase of *Penicillium* showing no specificity toward its substrates (ethyl acetate to olive oil).

Cholesterol esterification.—Sperry (284) states that esterification of free cholesterol occurs in sterile blood sera, the effect being abolished at 55° to 60°. The sterol may be esterified with palmitic, stearic, and oleic acids by the action of pancreatic lipase provided the sodium salt of a bile acid is present (285). Cholesterol esters are split by an enzyme present in normal serum but absent from cancer serum (286).

AMIDASES, ETC.

Glutaminase.—According to Krebs (287) the brain cortex and retina of vertebrates and the kidney of rabbits and guinea pigs can synthesise glutamine from glutamic acid and ammonia. The tissues which accomplish the synthesis possess a glutaminase breaking down glutamine to glutamic acid. The enzyme is specific and is inhibited by the *d*(—)-glutamic acid (the foreign enantiomorph) as well as by its optical isomer. Glutamic acid, however, does not inhibit liver glutaminase so that there exist two types of the enzyme, "brain" and "liver" types, having different pH optima and different relations to glutamic acid. They both differ from asparaginase.

Aspartase.—This specific enzyme which splits aspartic acid into fumaric acid and ammonia and which was first shown by Quastel & Woolf (288) to exist in bacteria is to be found in plants and may account, according to Virtanen & Laine (289), for the large yield of aspartic acid found among the amino acids formed by inoculated cultures of leguminous plants. It may also be partly responsible for variations in succinic acid formation in *B. coli* fermentations (325). The variation with temperature of the equilibrium constant for the reaction between fumaric acid and ammonia in presence of aspartase has been investigated by Jacobsohn & Tapadinhas (290).

Arginase.—Baldwin (291), who uses a manometric method for arginase estimation, states that the arginase content of a given tissue depends upon its physiological condition, the enzyme tending to disappear in starvation. A micromethod of determining arginase is described by Linderstrøm-Lang *et al.* (292).

Urease.—Grabar & Riegert's ultrafiltration studies (293) indicate that Sumner's crystalline urease in aqueous solution is the most homogeneous preparation studied, having dimensions near those of serum

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170

145950

globulins. Trypsin progressively destroys urease activity until the smallest molecules so produced are inactive.

Histaminase.—According to McHenry & Gavin (294) histaminase, prepared from kidney, splits ammonia only from the side chain of histamine, the rupture of the iminazole nucleus yielding no amino compounds.

Uricase.—Kleinmann states that 50 to 70 per cent of the uric acid in blood may be decomposed by uricase, aëration of the blood not being necessary (295). Kidney extracts possess the power of anaërobic uricolysis, no allantoin being produced (296).

NUCLEOSIDASES, ETC.

Adenosine is rapidly decomposed by nucleosidase and nucleodeamidase of beef kidney (297). A nucleosidase, specific for purine nucleosides, and not for pyrimidine nucleosides, may be obtained by drying frozen organs, adsorbing an extract on alumina and eluting with sodium arsenate (298). Guanine and hypoxanthine are strong inhibitors of the enzyme. Purine nucleosidases of various organs (spleen, lung, liver, cardiac muscle) are identical. A specific pyrimidine nucleosidase may also be obtained. Makino (299) describes a polynucleotidase which acts on thymus nucleic acid and exerts no nucleophosphatase or nucleosidase activities.

Two lecithinases are described by Contardi & Ercoli (300) in extracts of rice and *Aspergillus oryzae*, one of which splits lecithin into fatty acid and lysolecithin and the other which splits lecithin into fatty acid and choline glycerophosphate. The latter is split by a choline phosphatase into choline and glycerophosphate. Hughes (301) states that the rate of hydrolysis of a unimolecular film of lecithin to lysolecithin by venom lecithinase is dependent on pH, surface concentration of lecithin molecules, and the venom concentration. The enzyme is stable to prolonged boiling at pH 5.9 but is destroyed on boiling in solutions more alkaline than pH 7.0.

OTHER ENZYMES

Carboxylase.—Carboxylase of yeast may be stabilised by suspending dry yeast in three volumes of 50 per cent glycerol and allowing it to digest at 37° for five hours. The centrifuged fluid will retain its activity for a month. Carboxylase cannot be removed from the glycerol preparation by adsorption (302).

Fumarase.—The observation (303) that fumarase exists in tumours is confirmed (76). The thermodynamics of the fumarase system has been extensively studied by Jacobsohn & Soares (304).

Endosomase.—This enzyme which partially hydrolyses nucleoprotein and which is distinct from nucleases, nucleotidases, and proteases, exists in aqueous extracts of lymphatic ganglia of cattle (305).

Inositol oxidase.—This enzyme exists in brain, heart, kidney, and liver of rats, the brain being richest (306).

A mechanism for transforming tyramine (not phenylalanine or tyrosine) into adrenaline occurs in the medulla of adrenal glands (307). Decarboxylation of tyrosine to tyramine takes place in the kidney.

According to Guha & Ghosh (308), spleen, kidney, and liver tissue will form ascorbic acid from mannose, but not from glucose, fructose, galactose, arabinose, or xylose. They report that brain, heart, and leg muscle of the rat also have this power.

Chondrosulphatase.—A chondrosulphatase obtained from *B. fluorescens* liq., splitting chondroitin sulphuric acid, is described by Neuberg & Cahill (309).

Azotase.—This is the name given by Burk, Lineweaver & Horner (319) to the specific enzyme system which exists in *Azotobacter* and which catalyses the fixation of nitrogen at ordinary temperatures and pressures. The particular enzyme in the system which combines directly with nitrogen is termed "nitrogenase." The kinetics of nitrogen consumption as a function of pH has been studied. The Michaelis constant of fixation, Km_N , equals 0.22 atm. and is independent of pH and of the concentration of calcium or strontium though calcium is important for the fixation process.

Tryptophanase.—This name is given by Happold & Hoyle (310) to a specific enzyme in *B. coli* which transforms tryptophane into indole and has an optimum pH of 8.5. The enzyme is formed in greatest abundance when the organism is grown on a tryptophane-rich medium. Woods (311) has also investigated indole production from *l*-tryptophane in presence of resting *B. coli*. *d*-Tryptophane is not appreciably attacked. Under anaërobic conditions β -indole-propionic acid is formed.

Miscellaneous bacterial enzymes.—The conversion of *l*-proline into *d*-amino-*n*-valeric acid (in presence of alanine) by *B. sporogenes* is noted by Stickland (312).

Dubos (313) has examined the mechanism of production of a

specific enzyme decomposing capsular polysaccharides of Type-III pneumococcus. A maximum yield of the enzyme was found to be determined by the concentration of polysaccharides in the culture medium and by the amount of inoculum.

In the S and R forms of Aerttryche's bacillus and Gaertner's bacillus there is an enzyme which splits complete antigens of both species (314). It splits off the fatty acid of the toxic antigen to form a residual non-toxic antigen. The enzyme is rapidly attacked by activated trypsin.

Bottomley, Cavanagh & Polanyi (315) have investigated the rate at which atoms of deuterium are exchanged for hydrogen atoms of water in presence of resting bacteria (*B. acidi lactici*), the catalytic agent being an enzyme which is equally active whether the cells are dead or alive (probably hydrogenase).

ENZYME MODELS

Tamamuchi & Umezawa (316) state that with the aid of animal charcoal as a catalyst it is possible to obtain the reversible reaction: Succinic acid + methylene blue \rightleftharpoons fumaric acid + leucomethylene blue. Quinone and cyanide retard the reaction. Bredig *et al.* (317) have investigated the condensing action of diethylaminocellulose catalysts on benzaldehyde and cyanide with the formation of optically active oxynitriles. Other aldehydes were also investigated. Langenbeck (318) has discussed the general question of formation of artificial carboxylases and esterases.

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BIOCHEMICAL LABORATORY
CARDIFF CITY MENTAL HOSPITAL
WHITCHURCH, GLAMORGAN

X-RAY STUDIES ON THE STRUCTURE OF COMPOUNDS OF BIOCHEMICAL INTEREST*

BY O. L. SPONSLER

University of California at Los Angeles

AND

W. H. DORE

University of California

Agricultural Experiment Station, Berkeley, California

Investigations of crystal structure, when carried to completion, should give full information regarding the organization of a substance in the solid state. They should reveal not only the dimensions and shape of the unit crystal cell but also the space positions of each constituent atom in the unit. For such crystalline substances as the sugars and amino acids complete knowledge of the crystal structure would show the arrangement of the atoms within the molecule as well as the arrangement of the molecules within the crystal; but for substances such as the polysaccharides and the proteins, in which a less regular arrangement of the atoms is accompanied by the lack of a common crystalline appearance, such complete knowledge is not to be hoped for.

From the biochemical viewpoint, perhaps the chief interest in the crystal structure data lies in suggestions regarding the steric configuration of the constituent molecules of the crystal. It may be inferred that the molecule as it exists in the crystal is of the same size and shape as the free molecule in solution, except as allowance may need be made for distortions caused by crystal forces; *a priori*, comparatively slight distortions would be expected. Accordingly, complete knowledge of the structure of a given biochemical substance should afford valuable information as to its molecular stereochemistry and should play an important rôle in the solution of such vexing problems as the mechanism of optical rotation and the existence of various ring types among the sugars.

The contributions which crystalline or pseudo-crystalline structure can make to our knowledge of the high-molecular substances are related to such important properties as swelling, hydration, peptization, etc., which are due to the structure of the materials perhaps as much as to their chemical composition. In addition, indications may be

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obtained regarding micelles or crystallites and, if such exist, their dimensions may be revealed.

The extent and value of crystal-structure information depends upon the completeness with which the structure may be made known. Up to the present time complete crystal structures are known for only a few organic substances and most of these are not of direct biochemical interest. Many partial structure analyses, however, have been made of biochemical substances. Complete structure analyses are at present within the realm of possibility for only those biochemical substances which have molecules composed of relatively few atoms, although a method is in the process of being developed (1) which promises much greater latitude. Incompleteness here is not due to lack of zeal or effort on the part of the investigators, but as indicated is due to inherent technical difficulties. Among these the large number of parameters required to describe the positions of the numerous individual atoms, and the low symmetry of both the chemical molecule and the crystal combine to render complete analysis from x-ray data alone exceedingly difficult.

Partial structure analyses often go so far as to determine the unit cell of the crystal and the space group from which deductions concerning the molecule may be made. From this point of view, indirect solutions are sometimes attempted by the so-called "stochastic method" (2), in which, by using existing knowledge concerning the chemical nature of the molecule, and applying certain assumptions as to interatomic distances, valence angles, etc., a plausible space structure for the molecule may be found. After x-ray investigations have established the size of the unit cell, the number of molecules in the cell and the space group, an arrangement of the molecules is sought which is consistent with the cell dimensions and the symmetry requirements. The correctness of the proposed structure is tested by comparing the intensities observed for the x-ray reflections from the various planes in the crystal with the theoretical intensity of the corresponding reflections as calculated from the hypothetical structure. The method is used less often than might be expected because of ambiguities as to the probable steric configuration of the molecule, uncertainties as to intermolecular distances, and lack of accurate experimental intensity values.

Comparisons of cell dimensions and molecular dimensions are often instructive, but pitfalls may be encountered in making deductions from incompletely determined structures. Misleading conclusions may re-

sult from inappropriate selection of cell boundaries, erroneous conceptions of the steric configuration of the molecule, or erroneous assumptions as to the requirements of molecular packing for a given molecule. For example, Hendricks (3) has commented on the relationship between molecular orientation and cell dimensions and has emphasized the ambiguity which exists in regard to the cell boundaries of monoclinic crystals. Andress & Reinhardt (4) erroneously concluded from dimensional comparisons that sugar molecules in the crystal state are open chain molecules, but, as has been pointed out by Cox (5), this view is refuted by chemical evidence and by their x-ray data.

SUGARS

Information as to cell dimensions, space-group classification, etc., is available for about sixty crystalline sugars and related substances, due to the efforts of Cox and his associates (6, 7, 8, 9, 10, 10a), and to contributions from numerous other workers (11, 12, 13, 14, 15, 16). It is significant that the structure of no substance in the sugar group has been completely established. Tentative suggestions have been made as to the probable general structural organization of a number of the sugars and several proposed structures have been described in considerable detail. In no instance, however, have parameters for the constituent atoms been given, nor has the correctness of a structure been proved by rigid intensity comparisons.

A number of earlier investigators reported cell dimensions for sugars and their derivatives. Apparently the first serious attempts to work out molecular orientations in the unit cells of sugar crystals were made by Astbury & Marwick (17) who

formed the conclusion that the six-atom sugar ring is associated in the crystalline state with certain linear dimensions and that at least one of these dimensions usually corresponds to one of the axial lengths of the unit cell.

Based upon cell dimensions then available from various sources for two forms of cellulose, and four sugars, the conclusion was reached that the dimensions of the sugar ring have average values in three rectangular directions, of about 4.5 Å normal to the ring, 5.5 Å across the ring in a line with carbon atoms 1 and 4, and 7.5 Å across the ring in the direction of the side chain which is attached to the fifth carbon atom.

These dimensions are admittedly only approximate values and it is obvious from the data used that there is considerable individual

variation among the dimensions of the substances studied. Nevertheless, a knowledge of even approximate dimensions of the typical six-membered-ring hexose unit yields a constructive suggestion as to the way that the volume of a given unit cell may be partitioned among the several chemical molecules. Apportionment of the space to the separate molecules might well be expected to prove a first step in the successful deduction of the complete crystal structure. Unfortunately, experience has not fulfilled that expectation. As has been pointed out by Cox (6), "considerations of space-filling alone are not enough to decide between the various possible structures." Because of uncertainties as to the interatomic distances involved, "almost any of possible models for a sugar molecule may be made to fit" these dimensions.

One of the serious obstacles encountered in crystal-structure investigations of the sugars is the uncertainty as to the steric configuration of the six-membered ring sugar molecule. The various possible geometric forms of the six-membered sugar ring have been discussed at length by Haworth (18). If all of the atoms are coplanar, the valence bonds of the carbon atoms would make angles slightly less than 120° while the oxygen bonds would make a slightly greater angle. On the other hand, if the space model follows the Beyer plan of a strainless ring with a tetrahedral arrangement of the bonds, the bond angles will be about 109° and the six atoms cannot lie in a single plane. Three types of strainless ring models require consideration: (a) a *trans* or zigzag form in which carbon atoms 1, 3, and 5 lie in one plane while the ring-oxygen atom and carbon atoms 2 and 4 lie in another plane almost parallel to it; (b) a *cis* or cradle-shaped form in which four atoms lie in one plane while the other two atoms, in para-like positions in the ring, both lie on the same side of the plane of the other atoms; finally, (c), a form in which the five carbon atoms are coplanar while the ring oxygen is not in the same plane. For a given individual sugar ten steric forms are possible, viz., two *trans* forms, six *cis* forms and two forms of type *c*. Since the volumes and dimensions of these different types are of similar magnitudes, the approximate values given by Astbury & Marwick for the six-membered sugar ring do not afford a reliable basis for distinguishing between the various geometric isomers which would be possible for a given unit cell.

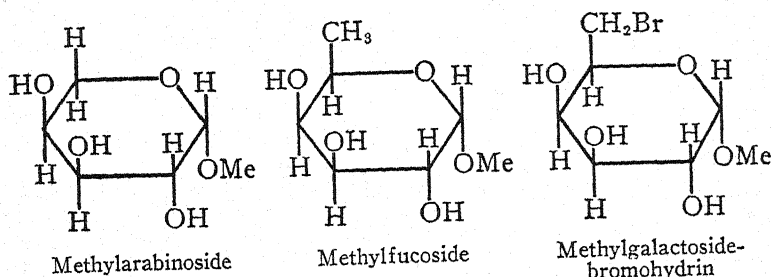
X-ray examinations of a number of sugars and their glycosides were made by Cox and his associates (6, 7, 8, 9, 10). Data were accumulated for a series of chemically related individuals in the hope that comparisons might divulge information as to size and mode of attach-

ment of substituted groups. The first studies were made on the pentoses and corresponding pentosides as the simplest prototypes of six-membered-ring sugar molecules. Of the possible geometric configurations of the pyranose ring, Cox (6, 7, 9) considered that the ring structure with five coplanar carbon atoms fits best in the cell dimensions of β -l-arabinose, α -d-xylose, β -methylxyloside, and α -methylxyloside. He proposed a structure for a α -methylxyloside for which he made partial intensity comparisons and which he regards as a fairly close approximation to the true structure. The unit cell is monoclinic and contains four molecules arranged with the ring planes parallel to the shorter diagonal of the inclined axes [i.e., parallel to the $(10\bar{1})$ plane]. Tentative structures were also proposed for the other pentose derivatives which he examined, but these structures were advanced with the reservation that they represent only rough approximations.

Cox & Goodwin (8) have examined the furanose and pyranose forms of α -methylmannoside; tentative structures were proposed for each. A puckered *trans*-ring structure was at first considered to be consistent with the data for the furanose unit but in a later communication Cox (9) stated that "the results for α -methylmannopyranoside are . . . unfavorable for a *trans* strainless ring." The furanoside would be expected to have a ring with all of its atoms coplanar. The data are consistent with this condition and seem to suggest that the side chain, $\text{CH}(\text{OH}) \cdot \text{CH}_2\text{OH}$, lies approximately in the same plane as the ring. This steric configuration for the furanose molecule departs startlingly from the older conventional stereochemical concepts. The free molecule may have possessed the conventional form with tetrahedral bond angles, becoming distorted by crystal forces when it became a building unit in the crystal.

In a recent paper, Cox, Goodwin & Wagstaff (10) presented a preliminary communication of what is to be a comparative survey of the cell dimensions of the various sugars that have been reported. It was the purpose of this survey to derive important general conclusions which can be applied later for the closer determination of structure in special cases. Cell dimensions are given for several sugars not previously reported. An example of morphotropism is pointed out in the comparative dimensions of the unit cells representing β -methylarabinoside, α -methylfucoside, and α -methylgalactoside-6-bromohydrin. The molecular structures of these substances have been definitely established by chemical evidence and it is known that they differ only in the groups attached to the fifth carbon atom. Their crystal lattices are

very similar with respect to the dimensions along the *b* and *c* axes, but the distances between the planes parallel to both *b* and *c* axes [the (100) planes] show differences which are consistent with the assumption that this is the direction that is obliged to accommodate the various groups on the fifth carbon atom. In the arabinoside, only hydrogen atoms are in this position and the packing of the molecules is the closest possible, corresponding to a distance of 8.1 Å between the planes. In the fucoside, where a hydrogen atom of the arabinoside is replaced by a methyl group, the corresponding interplanar distance has become 9.96 Å, an increase of 1.86 Å. Finally, in the bromhydrin, the CH₂Br group requires still more space and the interplanar spacing becomes 10.58 Å. The relation between the molecular constitution of these substances is shown in the following formulae:



The conclusion, that the change in dimension along one axis is that required by the groups on the fifth carbon atom, is supported by cleavage data. All three substances show cleavage parallel to the (100) planes, and this cleavage is more marked in the two compounds which have the larger attached groups. Consistent with this interpretation, the planes of the molecular rings are believed to be approximately parallel to the (100) planes and to be bonded together into sheets through their hydroxyl groups.

When complete and unambiguous determinations of structure have been made for a few members of the sugar group, it may be expected that the principles will be revealed which underlie the geometric configuration of sugar molecules and the bonding between molecules. The general nature of intermolecular secondary bonding may be anticipated to be a bonding of two oxygen atoms through a hydrogen atom. This type of union was first described by Huggins (19); it is believed to exist in ice crystals (20), acid potassium phosphate (21), boric acid (22), and oxalic acid (23). The hydroxyl groups in the sugars afford

abundant opportunity for the formation of O-H-O bonds, and it appears probable that such secondary valence attractions are the forces which are most important in binding together the molecules in the sugar crystals.

In a very recent paper, Cox, Goodwin & Wagstaff (10a) have reported x-ray data for twenty methylated sugar derivatives which afford additional evidence for the view that the pyranose ring is "flat" rather than puckered, with the five carbon atoms nearly coplanar but with the oxygen atom not in the plane with the carbons. It is pointed out that on theoretical grounds a greater angle than the normal tetrahedral angle should be expected in the sugar molecules; consequently it should be possible for the carbon atoms to be essentially coplanar and still have a strainless ring. Experimental support for this view is found in the comparative x-ray data for the methylated sugars. The cell dimensions indicate that the methylated sugar molecules have molecular "thicknesses" of about 4.5 Å. Careful study of the implications or the choice of any one of the possible geometric models of the ring indicate that the "flat" ring model gives the most satisfactory explanation of the observed data.

The authors conclude that their x-ray data give direct evidence as to the relative configuration of α - and β -glucose and α - and β -xylose. The commonly accepted structural formulae for these sugars are confirmed.

CELLULOSE

Several early workers in the field of x-ray crystal analysis showed cellulose to be crystalline or pseudo-crystalline and not amorphous as sometimes previously believed. The essential structural characteristics of cellulose were established (24, 25) as a bundle of long chains of chemically united glucose residues with the chains running in a direction approximately parallel to the fiber length. The cell dimensions given by different investigators are essentially alike, apparent differences being caused by a different choice of boundaries for the same unit cell (26, 27). The work of recent years has shown by convincing chemical evidence (28) that cellobiose exists preformed in cellulose and the earlier conclusion of Sponsler & Dore (24) regarding the union between glucose residues has been revised in favor of a chain structure that will accommodate the cellobiose linkage (33). The problem of accounting for the odd order reflections of the long identity period still remains unsolved. These and other investigations (31)

have revealed the general organization of cellulose, but exact details are still lacking and may be expected to remain so until the structures of some of the related simple sugars have been fully worked out. All assignments which have been made of exact atomic positions in the cellulose lattice must be regarded as speculative.

The definite establishment of the fine structure of cellulose as an orderly arrangement of parallel chains of glucose units has led to numerous attempts to determine the somewhat grosser, but still submicroscopic, organization of the structural units. Although the usual range of effective exploration by x-ray measurements is in the neighborhood of 1 to 10 Ångstrom units, i.e., from 0.0001 to 0.001 of a micron, it has been found possible to apply x-ray methods to the study of the larger organization of cellulose, and to obtain evidence bearing upon the reality and the possible size of micellar structural units. Logically, such studies upon the submicroscopic structure are followed by histological investigations which attempt to find reasonable correlations of the sub-microscopic organization and the microscopically visible anatomy of native cellulose fibers. An extended discussion of these studies would be out of place in this review; nevertheless, it appears desirable to note briefly some recent work in the histological field which constitutes an important extension of conclusions concerning the fine structure of cellulose.

The viewpoint was tentatively advanced by Sponsler & Dore (24) that the cellulose chains are of indefinite length. Staudinger (29) has favored the view of a very long cellulose chain, the so-called macromolecule, which, he concluded from viscosity measurements, is about 500 to 1000 glucose units long.

According to Hengstenberg & Mark (30) the chains are shorter and are arranged in bundles which constitute a crystallite or micellar unit, the fiber being made up of these crystalline units imbedded in non-crystalline material. The dimensions of the crystallite as calculated from measurements of the breadth of the x-ray reflections (27a) are reported as 500 Å in the direction of the fiber length and 50×50 Å in cross section. Clark (31) confirmed these values by direct measurement of the identity periods using a magnesium-target x-ray tube. Independent chemical evidence for the length of the cellulose chain was obtained by Haworth & Machemer (32) who determined the percentage of terminal tetramethyl glucose units in the mixture of products obtained from the hydrolysis of fully methylated cellulose, ascertaining thereby that the cellulose chain has a minimum length correspond-

ing to 100 to 200 glucose units, in satisfactory agreement with the values found by Hengstenberg & Mark and by Clark.

In spite of the agreement of three independent lines of evidence, there are reasons for doubting the real existence of micellar particles of the dimensions given. Sponsler (33) has pointed out that irregularities along a continuous layer may give rise to diffraction effects which might be erroneously interpreted to signify the actual discontinuity required by a micellar unit. This ambiguity of interpretation would apply to the results of Meyer & Mark as well as to those of Clark. The chemical evidence of Haworth & Machemer establish minimum values only for the chain length; the molecular chain in the native fibers may be many times larger.

Farr and associates (34, 35, 36; see also Clark [37]) have recently reported the isolation of ellipsoidal particles 1.5μ in length and 1.1μ in diameter by treatment of cellulosic fibers with pectin solvents. Microchemical tests indicate that these particles are composed of cellulose and are free from pectic material, and upon x-ray exposure they give the same diffraction pattern as given by untreated cellulose, but with much sharper lines. Since the particles, which are many times larger than the micellar units heretofore described, resist all attempts to break them down to simpler units by either chemical or mechanical methods, it is concluded that there is no evidence for the existence of smaller micellar units, and that these particles, and not the micelles, are the actual structural units of cellulose.

Sharp issue has been taken by Bailey & Kerr (38) with the conclusions of Farr and her associates. From microchemical studies the conclusion is drawn that the ellipsoidal particles described by Farr have no existence in the original plant tissue, but that, contrariwise, they are to be regarded as fragments produced by the degradation of cellulose by the drastic treatments which were used for their isolation. Accordingly, just as doubt has been cast upon the existence of micellar units in cellulose fibers, the reality of the larger postulated structural unit, the ellipsoidal particle, has in turn been called into question. The integrated contributions of histological, chemical, and x-ray investigations apparently leaves open the entire problem of the supercrystalline organization of the cellulose fiber.

AMINO ACIDS AND PROTEINS

A relationship somewhat similar to that between the simple sugars and the polysaccharides exists between the amino acids and the pro-

teins. In both cases chain structures are built from smaller units; in the latter, however, a knowledge of the atomic structure of one unit may be of relatively little aid in gaining an understanding of other units since many different kinds of amino acids are component units of a single protein molecule. Nevertheless two articles (39, 40) point out the desirability of a complete crystal analysis of the amino acids as preliminary to further studies of the larger molecules composed of amino acid residues. Realizing the difficulties of a rigorous method such as that applied to silicates by Bragg and West, the authors, following two somewhat different lines of procedure, lead, however, toward the same end, namely, the exact position of the atom. In Bernal's (39) report the results of the structure studies of some fifteen substances are given as preliminary to further details of atomic position, reports concerning which have not yet appeared.

Oscillation photographs were made from single crystals of α - and β -glycine, *l*-cystine, *d*- and *dl*-alanine, *d*-phenylalanine, *l*-aspartic acid, asparagine, glutamic acid, diketopiperazine; α -, β -, and γ -glycylglycine; and the barium chloride salt of glycine. For each of these, the space group and the size of the elementary cell were determined, and an attempt was made to fit molecular structures to them. Several different types of information were employed in essaying these attempts.

Making use of the accepted chemical formulae and the known radii of C, N, and O, and assuming the tetrahedral arrangement to be approximately correct, models were fitted to the unit cells according to the proper space group assigned, respectively, to the various substances. Other assumptions were made in order to bring the final structures into agreement with known properties of both the crystal and of the molecule. It was assumed that the molecule was an electrical dipole or polypole; that the molecules would so arrange themselves as to give a minimum electrical moment, and that the distances between neighboring molecules would be those of ionic packing. Optical and mechanical properties were called upon, and the final structures were tested for agreement between calculated and experimental intensities of x-ray reflections from the principal planes of the crystals. Diagrams of the positions and shapes were given for several amino acids and for one dipeptide. The results are frankly preliminary preparations for studies of exact atomic positions which are of course the important thing since all of the properties mentioned above are due to the atomic relationships in the molecule. His conclusions indicate that the molecules pack together in double molecules or in extended chains with

the dipole group as the determining factor. In aspartic acid the carbon chain seems straight, in asparagine and glutamic acid unassociated ring-like molecules are indicated.

The second paper, that by Hengstenberg & Lenel (40) gives the results of an attempt to use a more rigorous method in an analysis of only one amino acid, the simplest for the purpose, glycine. The method was that used by Bragg and West with silicates, but modified somewhat to fit the lighter atoms of the amino acids. It is based upon a determination of the variation in electron density throughout the crystal. The regions of greatest density are those in the immediate vicinity of the atoms, and by locating these regions the positions of the atoms are determined. The method, while fairly direct, is laborious and complicated, and has been used successfully for the determination of atomic positions where the molecules are composed of atoms which have considerable variation respectively in weight. Where the atoms are so nearly alike as are carbon, oxygen, and nitrogen of the amino acids, the results are less reliable. Recognizing the difficulties involved in the investigations, Hengstenberg & Lenel (40) nevertheless made a remarkably good attempt and although the co-ordinates they determined for the two carbons, two oxygens, and the nitrogen of the molecule are scarcely to be accepted as sufficiently accurate to be considered as final, they show the general form of the molecule as being similar to a model constructed from the accepted dimensions of atomic radii and their tetrahedral directional valences. The molecule built on their co-ordinates is considerably distorted although much less so than when built from an alternative set which they determined for the oxygen atoms. In the latter the carboxyl oxygens occur nearly in a plane with the carbons, both nearly equidistant from the carboxyl carbon and separated by a distance which is somewhat less than one would expect if the molecule were a zwitterion. The space group assigned to glycine by both Bernal (39) and these authors requires a paired arrangement of molecules in the crystal such as might result from pairing zwitterions. The elementary cell dimensions of the glycine employed by Hengstenberg & Lenel (40) are those of α -glycine of Bernal (39).

The difficulty attending a complete atomic analysis increases rapidly with increase in number of atoms in the molecule, especially when the atoms have about equal reflecting ability. This has militated against studies of the amino acids and has led investigators to make use of data from other sources to supplement that from x-ray diagrams. Further, when the size of the molecule becomes increased by addi-

tion of units, as in the peptides, a size is soon reached where crystallographic evidence is no longer available.

A considerable number of peptides have been investigated (39, 41, 42, 87, 89). In the smaller molecular forms where crystals have been obtained, a few characteristics of the molecules have been deduced from the form of the elementary cells of the crystals and from their space groups. Bernal (39) speaks of the "trans" character of the diglycylglycine but since the dimensions do not agree with those of Lenel (42) as shown in the tabulation, it leaves considerable uncertainty as to the validity of the deductions.

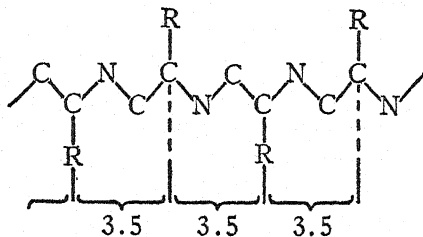
AXIAL DIMENSIONS OF DIGLYCYLGLYCINE CRYSTALS

	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
A diglycylglycine (Lenel)...	8.53 Å	4.3 Å	11.4 Å	105°30'
B diglycylglycine (Lenel)...	14.6	4.79	11.67	105°30'
diglycylglycine (Bernal)...	22.0	9.8	4.7	90°

On the other hand all of these authors speak of the peptides as chain molecules. Meyer & Go (41) studied peptides of glycine up to heptaglycylglycine and polyglycylglycines, which were, for the most part, not in a sufficiently crystalline state from which to obtain crystallographic data. The x-ray diagrams consisted of rings, similar to crystal powder diagrams, and from such diagrams interpretations become little more than inferences. A simple arrangement of chains, in planes spaced 4.15 Å apart, is indicated.

When, through addition of units, the molecule has increased to protein size a new arrangement frequently comes about in nature in which the long chain molecules are parallel and the units of the chain lose a great deal of their individuality. The resulting forms are fibrous structures; such for example as silk, hair, wool, feathers, quills, and scales. X-ray diagrams from these consist of a regular arrangement of spots which are not so clear-cut as from a simple crystal, but instead may be drawn out into arcs. Diagrams from the materials mentioned above have been studied by several investigators (43, 44, 45, 46, 47, 48, 49). They have found one dimension which is always associated with the long axis of the fiber and has been interpreted as the length of the peptide group in the chain since it checks well with a model built with the accepted atomic radii and the tetrahedral angles of carbon and nitrogen. This distance, about 3.5 Å, is repeated lengthwise of the

fiber and is found whenever the fiber is stretched out nearly to its elastic limit. The interpretation put upon this is that the protein molecule consists of a backbone of peptide groups to which the $-R$ of the amino acid is attached, alternating along the chain thus formed.



Two more dimensions are always found, which are associated with the cross section of the fiber. One, 4.5 \AA , is about the same in all proteins examined. The other varies, but in many proteins is about 10.5 \AA . Both are thought to be the lateral distances which separate the chain molecules. The zigzag backbones with the side chains form layers which are separated by 4.5 \AA , while the long molecules, which are parallel in the layer, are separated 10.5 \AA apart by the side chains. In silk this larger dimension does not occur but in its place a shorter one appears, since the side chains are practically all of glycine and alanine. In the glycine peptides of Meyer (41) the larger dimension is also absent. While in general the picture for silk (45, 46, 50, 51, 52, 53, 54) is one of single amino acids linked into peptide chains the details are still in question, for the x-ray diagrams are not the same when silk from different sources is used. Two types have been recognized for which more complete analyses are desirable.

A considerable number of proteins which do not form fibers nor crystals, as commonly recognized, have been investigated (55-76, 90, 98, 99). The usual diagram obtained from these is comparable to a powder diagram but is very diffuse, consisting of usually two or more rings. In most cases, where interpretation has been attempted, the two spacings 4.5 \AA and 10 \AA have been accepted as indicating the existence of chain molecules of protein in the sample, lying parallel to one another.

From studies of such diagrams Astbury & Lomax (77) observed a difference in the degree of crystallinity attained by a number of proteins and were led to develop a physical conception of hydration and denaturation.

Before denaturation has taken place the protein molecules seem to be clinging together chiefly through their side chains, but after denaturation there is a marked coalescence by way of the "backbone" spacing as shown by the sharpening of the 4.5 ring, and the appearance of a new reflection, especially when albumins were denatured by heat. This increasing degree of crystallinity was thought to express an increase in denaturation and a completely denatured state would be that in which the peptide chains were aggregated into crystallites. From this point of view the fibrous proteins, fibroin, gelatin, keratin, and myosin, may be considered as in a state of denaturation or disposed towards it. According to this interpretation all methods of denaturizing—heat, mechanical disturbance, radiation, acids, alkalis, etc.—lead to the break-up of a particular or "specific" arrangement, characteristic of the natural protein, in preparation for reaggregation into parallel bundles.

Proteins in crystalline form are being studied with renewed interest since it has been shown that water of crystallization is readily lost with resulting decomposition of the crystal (78, 79, 80).

Methods for obtaining very much larger interplanar spacings have presented new aspects for further study (81, 82, 83).

Several reviews of protein investigations have been published from time to time which include many theoretical and experimental details omitted here for lack of space (84-88, 91).

SOME OTHER ORGANIC COMPOUNDS OF BIOCHEMICAL INTEREST

In the last two or three years there has been marked activity in the investigation of the crystal structures of various biochemical preparations including sterols, hormones, enzymes, vitamins, pigments, and medicinal substances. Brief reference has been made to some of these by various authors in previous volumes of the *Review*. The results have led in most cases to the establishment of the dimensions of the unit crystal cell, and have afforded suggestions as to methods of molecular packing but have not established complete structures. The contributions of these studies to biochemical knowledge have been considerable in the aggregate, but somewhat fragmentary, usually in the direction of fixing the maximum dimensions of the molecular space model, and thereby deciding the reasonableness or unreasonableness of molecular constitutions which previously had been tentatively deduced from chemical evidence. In general, constructive information for the deduction of the molecular constitution has not followed directly from

x-ray investigations in this group; exceptions are to be noted in instances where molecular weight determinations from x-ray data have removed ambiguities as to the empirical formulae of the substances under consideration.

The investigations on the sterols and related substances by Bernal & Crowfoot (92, 93, 94) constitute an important contribution. The earlier papers reported cell dimensions for a number of the sterols and the closely related cardiac aglucones, and contained important suggestions as to the possible molecular constitution of ergosterol and other sterols (92). A more recent paper by Bernal & Crowfoot (93) describes the crystal-structure investigation of twenty-three hydrocarbons related to the sterols. These hydrocarbons are all based upon a phenanthrene nucleus and represent the skeletal framework of the sterols. Comparative data from these related substances give considerably more insight into the general structural relationships than it was possible to obtain from separate studies on a few individuals. Crystal-cell dimensions have been given and space-group assignments have been made for all of these hydrocarbons, and the shape and approximate dimensions of the constituent molecules of the crystal have been rather definitely indicated by these data. The molecules are described as lath shaped with cross sectional dimensions of about $6 \text{ \AA} \times 4 \text{ \AA}$, and with lengths varying with the number of condensed hydrocarbon rings (n) and expressed approximately by the formula: $(2.1n + 4.4) \text{ \AA}$. Although no atomic positions are given, typical arrangements of various hydrocarbons in their crystal lattices are described, and these structures are in good agreement with the structure of chrysene which has been fully worked out by Iball (95).

The crystal structures of the toad poisons, bufagin and cinobufagin, have been investigated by Crowfoot (94). The cell dimensions which were found for these substances indicate that their molecular structures are similar to those of the cardiac aglucones, as was anticipated from the similarity of their pharmacological action. Bufagin gave a molecular weight of 446 ± 9 , as calculated from the cell volume, in agreement with the new formula, $\text{C}_{24}\text{H}_{32}\text{O}_5$, which was proposed by Jensen (96) and in disagreement with older formulae. Calculations from the cell volume of cinobufagin indicate a molecular weight of 447 ± 10 and the author suggests that the formula is therefore $\text{C}_{26}\text{H}_{34}\text{O}_6$ (mol. wt. 442) rather than either $\text{C}_{25}\text{H}_{32}\text{O}_6$ (mol. wt. 428), which was advanced by Jensen & Evans (97), or the earlier formula $\text{C}_{29}\text{H}_{38}\text{O}_7$ (mol. wt. 507).

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UNIVERSITY OF CALIFORNIA AT LOS ANGELES

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UNIVERSITY OF CALIFORNIA
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BERKELEY, CALIFORNIA

THE CHEMISTRY OF THE CARBOHYDRATES AND THE GLUCOSIDES*

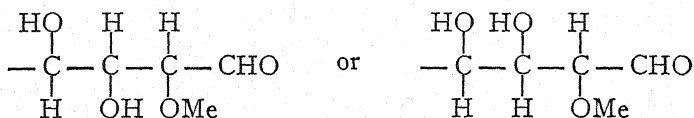
By W. N. HAWORTH AND E. L. HIRST

University of Birmingham, England

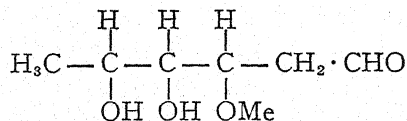
So many papers on the chemistry of the carbohydrates have been published during the period under review that only a very small proportion of them can be referred to in this article, and an endeavour has been made to select for comment groups of papers dealing in the main with problems of biochemical interest.

MONOSACCHARIDES AND DISACCHARIDES

General.—Further progress has been made in the constitutional study of digitalose, a methylated 6-deoxy-hexose which occurs in digitalis glycosides. Kiliani's early work seemed to have limited the choice of formula to four possibilities but it now appears that none of these can be correct. Since digitalose on oxidation yields a methylated derivative of *l*-trihydroxyglutaric acid the configuration of the second, third, and fourth carbon atoms must be either



The configuration of the fifth carbon atom is still undecided and choice has therefore to be made amongst four possible formulae (1). Cymarose is a methylated 2,6-bis-deoxy-hexose which occurs in strophanthin and other glucosides and oxidation reveals that the methoxy group is attached to the third carbon atom of the sugar chain (2). This result renders it likely that cymarose is 3-methyl-digitoxose,



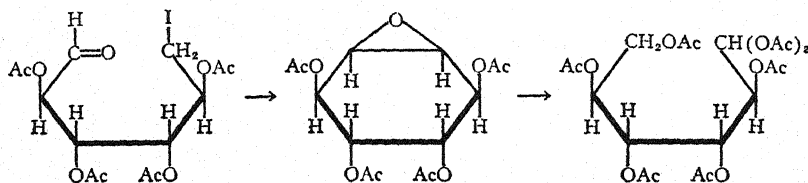
The sugar attached by glycosidic union to quercetin in the natural colouring matter rutin has now been proved to be a biose of rhamnose

* Received December 30, 1935.

and glucose with the constitution represented by β -*l*-rhamnopyranosido-6-*d*-glucopyranose (3).

The first example in the sugar series of a diketose is reported. The new sugar, 5-ketofructose, shares with the trioses and with *d*-glucose, *d*-fructose, *d*-mannose, and *d*-galactose in the monose series the property of being fermentable by yeast. The reasons for this behaviour are as yet little understood and further results in this field may be of considerable interest biochemically (4).

The chemistry of the cyclic alcohols is of special importance in view of the action of meso-inositol as a growth-promoting factor in the bios group. Nevertheless the direct transformation of hexoses into cyclic alcohols by aldol condensation has not been accomplished *in vitro* and for this reason some recent results recorded by Micheel and his collaborators are of particular interest. These authors find that the action of acetic anhydride and zinc chloride on 6-iodo 2,3,4,5-tetra-acetyl aldehydo-*d*-galactose results in the formation of hepta-acetyl aldehydo-*dl*-galactose.



No simple explanation of the formation of *dl*-galactose is possible and the authors consider it most probable that at an intermediate stage the anhydro cyclic alcohol derivative is formed. Subsequent opening of the ethylene-oxide ring of this symmetrical substance must then lead to the formation of a derivative of racemic *dl*-galactose (5).

Condensation of sugars with carbonyl compounds.—The acetone compounds of the sugars and analogous condensation products with acetaldehyde, benzaldehyde, furfuraldehyde are being increasingly employed for the preparation of partially substituted derivatives required for synthetic work, and results of great interest are emerging from a comparative study of the mode of condensation of these aldehydes and ketones. It would appear that condensation with acetone usually, but not invariably, involves *cis*-hydroxyl groups at contiguous carbon atoms and that in the case of the free sugars readjustment of the pyranose ring to the furanose form will occur when such a change

renders available two *cis*-hydroxyl groups. Well-known examples of these rules are found in the diacetone derivatives of glucose, galactose, mannose, and fructose, and a more recent example is provided by talose (6) which behaves like mannose. Accommodation of this type is impossible in the case of glycosides and unless suitably placed hydroxyl groups are present no condensation takes place, as for instance with α -methylglucoside. On the other hand the *cis*-hydroxyl groups at C₂ and C₃ in α -methylmannopyranoside readily react and both 2,3-monoacetone α -methylmannopyranoside and the 2,3,4,6-diacetone derivative are readily obtainable (7). The spanning of the 4,6-positions by acetone is unusual, but the same kind of linkage is found also in xylose diacetone (8) in 1,2,3,5-diacetone glucose (9) and in sorbose diacetone (10), and this type of condensation becomes of greater importance when the condensation of aldehydes with sugars is considered. It is important, in view of earlier claims to the contrary (11), to note that no ring change takes place during these condensation reactions unless circumstances are such that it will result in the ordinary way by recognised chemical transformations, as is the case, for instance, if the acetone employed contains methyl alcohol and an acid catalyst is used (7).

Normally, condensation with acetone spans contiguous hydroxyls, making a cyclic link of five atoms, but aldehydes usually span alternate hydroxyls and furnish a cyclic six-atom substituent to the sugar chain, which is found for example in 4,6-monobenzylidene glucopyranose (12). Moreover benzaldehyde, unlike acetone, condenses with both α - and β -methylglucopyranoside giving the corresponding 4,6-benzylidene derivative (13). Furanose derivatives are obtainable by condensation of benzaldehyde with substances in which the glucofuranose ring is already present, e.g., 1,2-monoacetone 3,5-benzylidene glucofuranose (14) and 1,2-monoacetone 5,6-benzylidene glucofuranose (15). In the few cases which have been studied acetaldehyde seems to resemble benzaldehyde rather than acetone in its mode of condensation. For example it condenses with glucopyranose (16), giving 4,6-ethylidene α -*d*-glucopyranose analogous with the 4,6-benzylidene derivative. Nevertheless the formation of tri-ethylidene sorbitol shows that five-membered rings are also possible (17). The ethylidene grouping is sometimes readily replaced by benzylidene on treatment of the substance with benzaldehyde and zinc chloride, e.g., 2,3-dimethyl 4,6-ethylidene β -methylglucopyranoside is transformed in this way into the corresponding 4,6-benzylidene derivative (16).

Furfuraldehyde behaves similarly to benzaldehyde giving the 4,6-monofurfurylidene derivative with α -methylglucopyranoside and the 2,3-4,6-difurfurylidene derivative with α -methylmannopyranoside (18). These oxo-derivatives of sugars facilitate the preparation of substances which are otherwise almost inaccessible. For instance, triethylidene sorbitol (17) and 2,4-benzylidene sorbitol (19) provide a means of access to *l*-xylose. Important uronic acids are obtainable by oxidation of acetone compounds in which position 6 is unsubstituted, e.g., galacturonic acid from galactose diacetone (20); mannuronic acid from 2,3-monoacetone α -methylmannopyranoside (21); glucuronic acid from 1,2-monoacetone 3,5-benzylidene glucofuranose (22). Keto acids are similarly obtainable, e.g., 2-keto-*d*-gluconic acid from fructopyranose 2,3-4,5-diacetone (23) and 2-keto-*l*-gulonic acid from 2,3-4,6-diacetone-*l*-sorbitol (10).

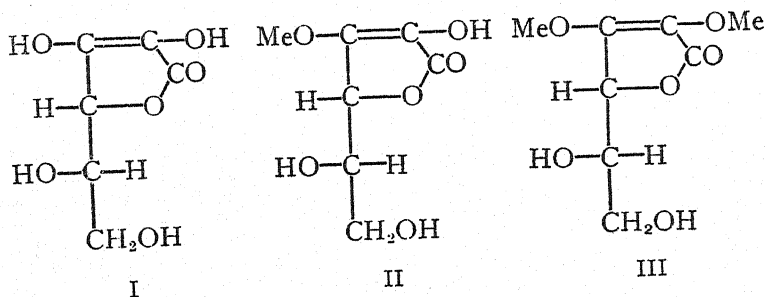
Walden inversion in the sugar group.—Walden inversions in the sugar series are of special biological importance in view of the glucose-galactose transformation and, although this particular interchange is difficult to imitate *in vitro*, considerable progress has been made in connection with Walden inversions which take place during the hydrolysis of *p*-toluenesulphonyl derivatives of sugars. When a free hydroxyl group is available on the contiguous carbon atom, removal of the *p*-toluenesulphonyl residue results in the formation of an anhydro ring which may then be ruptured by further and more drastic treatment. A study of the products of such reactions indicates that both the formation and rupture of the anhydro ring are usually accompanied by inversion. Thus, 2-benzoyl 3-*p*-toluenesulphonyl 4,6-benzylidene α -methyl-*d*-glucopyranoside gives with sodium methylate 4,6-benzylidene 2,3-anhydro α -methyl-*d*-allopyranoside (inversion at C₃) and further action of sodium methylate opens the anhydro ring with formation of a derivative of *d*-altrose (inversion at C₂). Similarly, the corresponding 2-*p*-toluenesulphonyl 3-benzoyl compound yields a 2,3-anhydromannose derivative (inversion at C₂) and then a derivative of *d*-altrose (inversion at C₃) (24). 2,3-Dibenzoyl 4-*p*-toluenesulphonyl 6-triphenylmethyl α -methyl-*d*-glucopyranoside when treated with alkali gives a 3,4-anhydro derivative of galactose (inversion at C₄) which is hydrolysed by dry hydrogen chloride in acetone to derivatives of *d*-gulose (inversion at C₃) and of *d*-galactose (no inversion) (25). A similar series of changes appears to occur when 2,3,6-triacetyl 4-*p*-toluenesulphonyl β -methyl-*d*-glucopyranoside is used (26). The 3,4-anhydride is probably a galactose derivative (inversion at C₄) and

on opening the ring derivatives of gulose are formed (inversion at C_3). No galactose was detectable in this instance but a derivative of *d*-glucose was observed (inversion at C_4). The behaviour of 3-*p*-toluenesulphonyl 1,2-monoacetone *d*-fructopyranose is similar. This gives a derivative of 3,4-anhydro *d*-psicose (inversion at C_3) and, by subsequent inversion at C_4 (by use of sodium methoxide), *d*-sorbose is produced (27). With sodium hydroxide, however, fructose is also obtained (inversion at C_3). When the toluenesulphonyl group is in position 6 (symmetrical grouping) anhydride formation is not accompanied by inversion and the resulting ethylene-oxide ring can then be broken in two ways, e.g., with the acetone derivatives of 5,6-anhydroglucose, acid hydrolysis yields glucose and alkaline hydrolysis a mixture of glucose and idose [inversion at C_5 (9)]. It is not yet clear whether anhydro ring formation is a necessary concomitant of Walden inversion or whether simple hydrolysis of the toluenesulphonyl group is sufficient to cause inversion. That ring formation may be a necessary condition is suggested by such facts as the quantitative hydrolysis of 3-*p*-toluenesulphonyl diacetone glucose to glucose, and the hydrolysis of 4-*p*-toluenesulphonyl 6-triphenylmethyl 2,3-dimethyl α -methylglucoside which proceeds without inversion (25). On the other hand it has been claimed that hydrolysis of 2,3-monoacetone 5-*p*-toluenesulphonyl methyl-*l*-rhamnofuranoside gives directly (no anhydride formation is possible) a derivative of 6-deoxy *d*-gulose (inversion at C_5) and that the corresponding 4-*p*-toluenesulphonyl derivative of methyl *l*-rhamnopyranoside gives a derivative of 6-deoxy *l*-talose [inversion at C_4 (28)]. The facts are disputed, however, by Levene & Compton, since the properties of synthetic 6-deoxy *d*-gulose are at variance with those of the substance obtained from *l*-rhamnose (29). In the circumstances, until these discrepancies are cleared up, it cannot be said that there is in the sugar series a clearly established case of inversion accompanying alkaline hydrolysis of a *p*-toluenesulphonyl group without anhydro ring formation.

In the above examples the anhydro ring is ethylene oxidic, and it appears that this type of ring is preferred even when other hydroxyl groups are available for ring formation, e.g., during the alkaline hydrolysis of 6-*p*-toluenesulphonyl 1,2-monoacetone glucofuranose (9). In the latter instance the much more stable 3,6-anhydro ring is formed only when the hydroxyl group at C_5 is protected as it is in 1,2-monoacetone 5,6-di-*p*-toluenesulphonyl glucose. Only the acyl group at C_6 is removed under the usual conditions of hydrolysis and

the product is a derivative of 3,6-anhydro-*d*-glucose (30). An interesting example of a five-membered ring is encountered in the anhydride formed by the alkaline hydrolysis of 4-*p*-toluenesulphonyl 2,3,6-trimethyl *d*-glucose. It is claimed that this 2,3,6-trimethyl 1,4-anhydrohexose furnishes on acid hydrolysis a derivative of *l*-idose, showing that at some stage a totally unexpected Walden inversion has occurred at C₅ (31). Reference may be made also to Walden inversions brought about by the action of aluminium trichloride on sugar acetates. Lactose octa-acetate gives in this way a disaccharide, neo-lactose, from which *d*-altrose is obtained on hydrolysis; inversion must therefore occur at C₂ and C₃ in the glucose half of the molecule (32).

*Ascorbic acid and its analogues.*¹—There has been continued activity in the study of ascorbic acid and its analogues. The formula (I) for *l*-ascorbic acid, proof of which was given early in 1933 (33), has now found universal acceptance and considerable progress has been made in the detailed examination of the chemical and physiological properties of substances containing this type of ring system. Although, remarkably enough, ascorbic acid does not undergo opening of the lactone ring in the presence of alkali, the 2,3-dimethyl deriva-



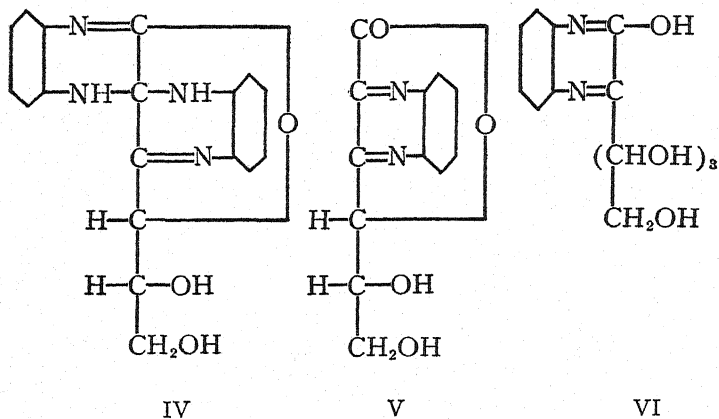
tive behaves normally and gives the sodium salt of the open-chain acid which, like some other Δ^{α} -unsaturated acids, resists the action of ozone. With the object of finding out which of the enolic hydroxyls in ascorbic acid is the more acidic, the action of limited quantities of diazomethane on the acid has been studied (34). Under these conditions the main product is the 3-methyl ether (II), which gives an intense blue colour with ferric chloride. It is apparent, therefore, that the enolic group at position 3 has the greater acidity. On further

¹ Cf. also this volume, p. 369. (EDITOR.)

methylation II gives crystalline 2,3-dimethyl ascorbic acid (III), which after complete methylation, followed by ozonisation, yields 3,4-dimethyl *l*-threonic acid unaccompanied by the epimeric 3,4-dimethyl *l*-erythronic acid. Since a sample of tetramethyl ascorbic acid obtained by direct methylation, without separation of the crystalline dimethyl derivative, gave both substances, confirmation is provided for the view that ascorbic acid can react in more than one structural modification (35). There is additional evidence in support of this in that methylation with diazomethane yields, besides II, a small quantity of an isomeric monomethyl derivative (?2-methyl ascorbic acid), which gives but a feeble fleeting colour with ferric chloride, and differs markedly in chemical and physical properties (including the absorption spectrum), from the 3-methyl ether. Furthermore, in contrast with the behaviour of the 3-methyl ether it gives no oxalic acid on ozonisation (35). The existence of tautomerism in di-enol systems of this type is well shown also in experiments on the action of diazomethane on thiochromonediols (36). In connection with the methyl ethers of ascorbic acid reference may be made to Micheel & Kraft's criticism (37) of the use of Weerman's reaction in structural investigations. The reaction was used in the proof of the structure of ascorbic acid to diagnose the presence of an α -hydroxy group in the dimethyl threonic acid obtained from tetramethyl ascorbic acid. The essential feature of the test is that α -hydroxy amides give sodium cyanate on treatment with sodium hypochlorite, whereas other amides do not. Careful investigation of various test cases has, however, completely substantiated the view that the test is specific for α -hydroxy amides and that this extremely useful method may be applied with confidence to distinguish between α -hydroxy and α -methoxy acid-amides (38).

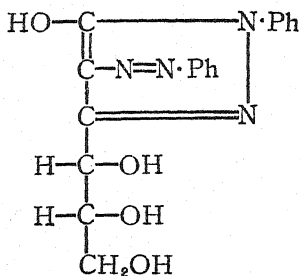
The *d*-glucosaccharosonic acid of Ohle and his collaborators (39), which is identical with Maurer & Schiedt's isovitamin C (40), has been shown to be the enantiomorph of *l*-arabo-ascorbic acid obtainable synthetically from *l*-arabinose (46). The *d*-isomeride has properties very similar to those of ascorbic acid, and it has been used by Ohle in investigating the action of *o*-phenylenediamine and phenylhydrazine on substances which possess the ring structure present in ascorbic acid. Both arabo-ascorbic acid and its primary oxidation product react with *o*-phenylenediamine giving IV, which in the presence of dilute mineral acid gives V. This behaves as a true lactone and readily gives the free acid and derivatives thereof (41). The lactone does not react with

o-phenylenediamine and, by way of contrast, we may refer to the substance VI obtained by the interaction of 2-ketogluconic acid and *o*-phenylenediamine (42). This substituted quinoxaline readily breaks down by fission of the molecule between C₃ and C₄, giving, for example, glycerol when acted upon by phenylhydrazine. Such a fission into a three-carbon derivative is of particular interest since such reactions may ultimately serve to throw light on similar well-known biological degradations.

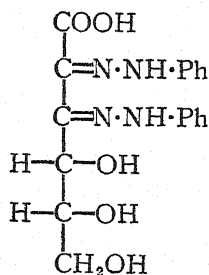


The nature of the isomerism displayed by the phenylhydrazine derivatives of *l*-ascorbic acid and *d*-arabo-ascorbic acid has been elucidated by Ohle and his collaborators. It had already been established that ascorbic acid condenses with two molecules of the base giving two well-defined isomeric substances both of which appear to be derived from the oxidised form of the acid (33). The relationship between these has now been cleared up as the result of studies with arabo-ascorbic acid (43). The position is complex, and only a summary of the final results can be given here. For proofs of the structure of the various derivatives the reader must consult the original papers. It appears that in the presence of acetic acid the product is mainly the substituted pyrazolone (VII), whereas with mineral acids only a little of VII is produced and a mixture of the acid (VIII) and the lactone (IX) is obtained. Substance VIII is easily transformed into IX and VII by simple recrystallisation and finally IX is converted by hot dilute alkali solution into the pyrazolone derivative (VII). Similar experiments with *l*-ascorbic acid indicate that the yellow derivative

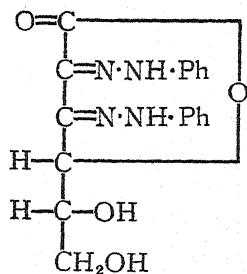
(m.p. 210°) has the pyrazolone structure corresponding to VII and that the red product (m.p. 197°) is the true osazone (structure as IX).



VII



VIII

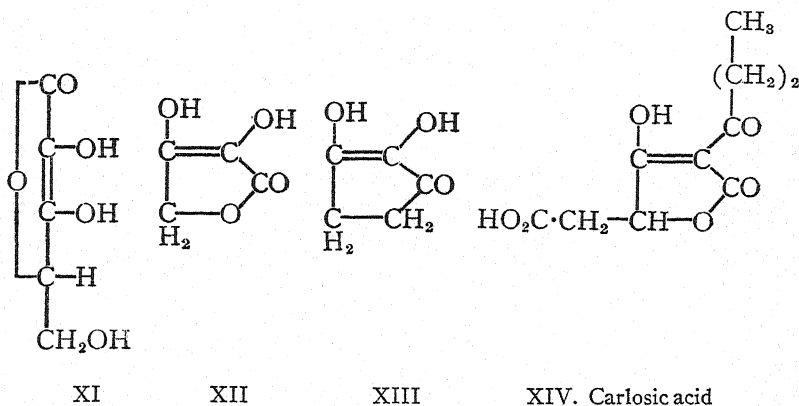


IX

Very rapid progress has been made in the synthesis of ascorbic acid and analogous substances. Three principal methods are now available: (a) addition of hydrogen cyanide to an osone followed by hydrolysis of the addition compound [Reichstein *et al.* (44, 47) and Haworth, Hirst, *et al.* (45, 46, 51)]; (b) the rearrangement of 2-keto 3,4-dihydroxy acids or their esters by the action of alkali [Ohle *et al.* (39) and Maurer & Schiedt (40)]; (c) oxidation of osones to 2-keto-acids followed by rearrangement as in method *b*. Methods *a* and *c* are restricted in scope by the inaccessibility of the necessary osones and *c* suffers further by reason of poor yields during the oxidation. When the osone required is available the first method is of great use and has already been utilised to obtain the following analogues of ascorbic acid, all of which have chemical properties and absorption spectra closely similar to those of natural ascorbic acid: *d*-gluco-ascorbic acid (45, 46, 47), *l*-gluco-ascorbic acid (48), *d*-galacto-ascorbic acid (46, 47), *l*-arabo-ascorbic acid (46, 47), *l*-gulo-ascorbic acid (47), *l*-allo-ascorbic acid (49), *l*-rhamno-ascorbic acid (50), *d*-xylo-ascorbic acid (*d*-ascorbic acid) (44, 45) and, in addition, the naturally occurring *l*-ascorbic acid (44, 45). (The nomenclature employed above derives the name of the substance from that of the osone used in the synthesis.)

The mechanism of the synthesis by the first method has been elucidated during a detailed investigation of the synthesis of *d*-gluco-ascorbic acid (51). The first stage consists in the formation of a crystalline addition product, $C_7H_{11}O_6N$, which displays a strong absorption band at 275 mμ. The chemical and physical properties of this substance show that this is not the expected open-chain cyanohydrin

with sodium methoxide (53). Other analogues of ascorbic acid which have been obtained by isomerisation of 2-keto-acids include *d*-arbo-ascorbic acid [from 2-keto *d*-gluconic acid (40)] and *l*-erythro-ascorbic acid [XI (54)] derived by a series of transformations from *l*-adonose, the last-named substance being produced in turn by the oxidative action of bacteria on adonitol (55). The simplest exact analogue



of ascorbic acid is XII obtained by Micheel & Jung (56) from the benzoyl derivative of methyl glycollate. Several other substances are known, however, which simulate in certain particulars the chemical behaviour of ascorbic acid. Many of these, and all the synthetic analogues, show absorption spectra having bands close to, or in the same position as, that of ascorbic acid. Amongst such substances may be mentioned (a) reductone (hydroxymethyl glyoxal), formed by the action of alkali on sugars (57), (b) reductic acid, XIII, prepared by the action of dilute sulphuric acid at high temperatures on various carbohydrates [glycuronic acid, galacturonic acid, xylose, etc. (58)], and (c) the remarkable series of substituted tetronic acids produced as metabolic products of *Penicillium Charlesii* grown on Czapek-Dox-glucose medium. The structure of these has been elucidated by Clutterbuck, Raistrick, & Reuter (59) and a typical member of the series is shown in XIV.

The above review shows that already a large number of closely related analogues of ascorbic acid is available for the investigation of the correlation between constitution and physiological activity. Synthetic *l*-ascorbic acid has the full activity of the natural product (60,

61). The primary oxidation product of *l*-ascorbic acid is also fully active (62) but the imino-ascorbic acid, obtained as an intermediate body during the synthesis from *l*-xylosone, has no antiscorbutic activity. *l*-Rhamno-ascorbic acid has about one-fifth (50), *d*-arabo-ascorbic acid about one-twentieth (60, 63), and *l*-gluco-ascorbic acid about one-fortieth (48, 64) the activity of *l*-ascorbic acid. *d*-Ascorbic acid, *d*-gluco-ascorbic acid, *d*-galacto-ascorbic acid (60, 64), reductone (57), and reductic acid (58) are all inactive and reference to the structural formulae shows that the active substances have formulae in which the ring engages a hydroxyl group on the right of the carbon chain of the conventional Fischer formula. The opposite is the case with the inactive compounds and suggestions have been made (48, 53) that the former type of configuration may be a necessary condition of antiscorbutic activity. Further progress in the elucidation of this problem has been reported recently by Zilva (64) who has considered the behaviour of these substances in relation to their retention by the animal organism.

Polysaccharides.—Although the main features of the structure of the principal polysaccharides may now be regarded as settled, special interest is still centred on problems concerned with such important matters as molecular size and conformation. Estimates of molecular weight derived by different methods (e.g., from viscosimetric measurements, osmotic pressure, ultracentrifuge data, and the gravimetric chemical assay by the end group method) are frequently at variance with one another. The discrepancies, although not yet fully explained, indicate that a clear distinction must be drawn between the chemical molecule and the variable physical unit which is present in solutions. It is highly probable, also, that many of the published measurements refer to substances in which the chemical (or physical) units are of a variety of different sizes and it is pointed out in a recent important paper that the observed molecular weight (which is necessarily an average value) must vary according to the method of measurement. Ordinary physicochemical methods lead to a number average, viscosity data yield a weight average and, besides both of these, the ultracentrifuge data provide a third kind of average—termed the *Z*-average. The results reveal the extreme power of the ultracentrifuge method of investigation and show that by its application detailed information can be obtained of the state of affairs in solutions containing molecules whose sizes vary over a wide range (65).

For instance the amylose and amylopectin fractions of starch have

chemical molecules consisting of some 26 to 30 glucose units and it was suggested that the essential difference between them lay in their state of aggregation into large physical units (66). Ultracentrifuge measurements now show that both amylose and amylopectin are poly-disperse, the former consisting largely of particles of "molecular weight" of some 60,000. For the latter the corresponding figure is about 300,000 (67). Viscosity measurements also indicate large aggregated particles but in such cases, particularly in the α -glucosidic series (starch and glycogen), the interpretation of viscosity data is far from simple. It is interesting therefore to find that starch can be disaggregated giving a particle which retains the full length of the chemical molecule but has a viscosity in agreement with the value calculated for the single molecules of chain length 26. This disaggregated starch is soluble in cold water but when heated or dried it retrogrades rapidly to the insoluble condition. The phenomenon of retrogradation is of frequent occurrence and it is held to be due to a combination and aggregation of chemical molecules together with dehydration (68).

Other starches besides potato starch, e.g., maize starch and waxy maize starch (69), have chemical molecules some 26 to 30 glucose units in length, although their properties and behaviour differ widely from those of potato starch. Examination of α -amylodextrin, prepared by the action of β -amylase on potato starch, shows that this dextrin retains the phosphorus content of the original starch, and although its chemical molecule is now one of 17 glucose units, viscosity data reveal that this shorter molecule still retains the capacity to aggregate (70). It has already been shown that the phosphorus content of a starch does not run parallel with the solubility in water. Nevertheless the phosphorus is an integral part of the substance and recent work indicates that the small nitrogen content of many starches may be equally important, and the suggestion is made that the gel-forming amylopectin of potato starch may be a flocculated co-acervate of amylophosphoric acid and one or more proteins (71). It is difficult, however, at present to see how this view can be reconciled with the behaviour of certain cereal starches which aggregate strongly (admittedly without the gel formation typical of potato starch) and yet possess extremely small phosphorus contents.

In this series of polysaccharides, glycogen [several samples of which of varying origin have been examined by the end group method, and have been found to possess a chain length of 12 to 14

glucose units (72)] furnishes solutions which are still typically colloidal although of low viscosity. Dextrins of smaller chain length (10 units and 7 units respectively) have been prepared from starch and at these chain lengths there is satisfactory agreement in the values for molecular weight obtained from the chemical method, the viscosity data, and rotational data (73).

Many papers have appeared dealing with the size of the cellulose molecule. Measurements by Stamm (74) and by Kraemer & Lansing (75), using the sedimentation-equilibrium method, indicate for native cellulose in cuprammonium a molecular weight of about 300,000 (i.e., nearly 2000 glucose units). There are difficulties, however, in that cuprammonium solutions are unstable and the figure quoted is arrived at by extrapolation. In addition, care in interpreting the results is necessary in that the observed value may be in reality a "particle" weight referring to aggregates of molecules. Similar caution is requisite in considering the value (750 to 900 units) arrived at by viscosity methods. Other difficulties arise also in connection with the viscosity method. For instance, the initial value for ramie in cuprammonium (1300 glucose units) falls to the constant value 900 and the reason for the decrease is unknown. Detailed examination has shown that cellulose dissolved in cuprammonium can be recovered with unchanged viscosity (corresponding to 900 units) if air and light are rigidly excluded from the solution (76). It follows that chemical breakdown can therefore be avoided in these solutions, but there remains a discrepancy between the ultracentrifuge and viscosity determinations of molecular (particle?) weight. It may be noted in this connection that, for reasons which are not yet properly understood, the viscosity of cellulose nitrate is abnormally large and that from viscosity data in *m*-cresol the "molecular weight" of methylated cellulose corresponds to a chain length almost twice that of the cellulose acetate used in the preparation of the methylated derivative. For possible explanations of these difficulties the reader is referred to the original papers (77, 78). In a recent paper Staudinger (79) compares the values derived from osmotic pressure measurements with those obtained viscosimetrically and, when due precautions are taken, finds good agreement in the case of cellulose, cellulose nitrate, cellulose acetate of various degrees of "degradation," and methyl cellulose (in aqueous solution). It is abundantly clear in view of these and other results that the macromolecules of cellulose are exceedingly large. These conclusions are in agreement with the long-established chemical

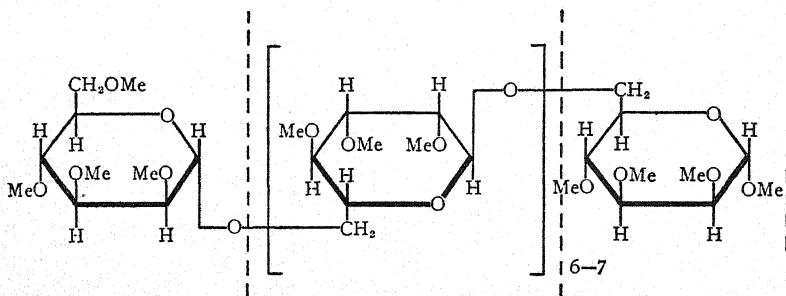
evidence concerning the structure of cellulose and it becomes all the more obvious that the low molecular weights postulated by Hess and his collaborators are abnormal and are not capable of direct interpretation in terms of molecular weight (80).

Polysaccharides based on fructose continue to give rise to problems of unusual interest. The levan produced by the action of *B. mesentericus* on sucrose consists of chains of fructofuranose units joined through positions 2 and 6 (81), in contrast with inulin where the junctions involve positions 1 and 2. It now appears that the chain length of this polysaccharide is about 10 fructose units. The determination is made by gravimetric assay of the end group which gives rise to tetramethyl fructofuranose after methylation of the polysaccharide and subsequent hydrolysis (82). A very similar polysaccharide, with a chain of fructofuranose units connected through C₂ and C₆, has been found in meadow grass and it becomes apparent that in plants reserve carbohydrates may be stored as chains of fructofuranose units in at least two ways: (a) as the inulin type (junction 1 and 2) and (b) as the levan type [junction 2, 6 (83)]. The occurrence of fructopyranose units in a natural polysaccharide has not yet been observed and it is of interest to find that studies on the formation of levan by bacterial action suggest that a potential fructofuranose residue must be present in any material convertible into levan (84).

Other fructofuranose polysaccharides which have received attention are irisin and graminin. Both these give methylated derivatives which on hydrolysis yield tetramethyl fructofuranose, a trimethyl fructose, and a dimethyl fructose in approximately equal proportions and it is suggested that graminin may contain a nucleus of four fructose residues with side chains (85). Schlubach & Schmidt (86) have examined inulins from different sources and find slight differences in rotatory power and rate of hydrolysis. The almost negligible reducing power of inulin is noted and the nature of the terminal potential reducing group is discussed in the light of possibilities outlined for other polysaccharides by Haworth, Hirst & Oliver (89).

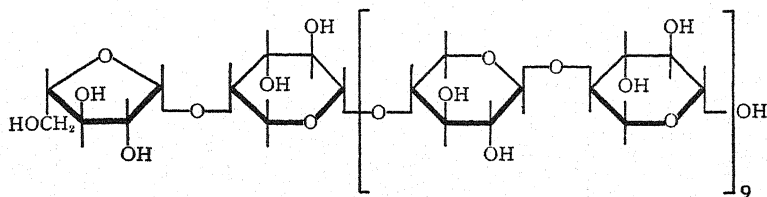
Progress is reported in the constitutional study of mannan-A, present in ivory nut. Hydrolysis of the methylated derivative yields 2,3,6-tri- and a small quantity (1 to 1.4 per cent) of 2,3,4,6-tetramethyl mannopyranose. By partial hydrolysis methylated mannobiose and mannotriose derivatives have been obtained and it appears that the polysaccharide consists of a terminated chain of some 80 consecutive units of mannopyranose linked through positions 1 and 4, as are

the glucose residues in cellulose (87). The constitution of another type of mannose polysaccharide is dealt with in the first of a series of papers concerned with carbohydrates synthesised by micro-organisms. The new substance is termed mannocarolose and is produced when *Penicillium Charlesii*, G. Smith, is grown on Czapek-Dox-glucose solutions. It yields only mannose on hydrolysis and its fully methylated derivative gives rise to tetramethyl mannopyranose (13 per cent), 2,3,4-trimethyl mannopyranose, and some 2,3-dimethyl mannose. Iodine values, viscosity measurements, and the chemical end-group assay all point to a chain length of about 9 units, and in this instance the mannopyranose residues are united through positions 1 and 6 as are the glucose residues in gentiobiose. With certain reservations due to the presence of dimethyl mannose in the hydrolysis products the simplest representation of the structure of the polysaccharide is that given below (88).



A new phase in the study of xylan, which occurs in woody tissue and forms some 30 per cent of esparto cellulose, has been opened up by the discovery that this polysaccharide is not composed entirely of xylose residues but contains combined arabinose (89). Investigation of the mode of linkage of the arabinose revealed that it was present as arabofuranose, this being the first recorded instance of the natural occurrence of an arabofuranose derivative. Since methylated xylan gives on hydrolysis 6 per cent of 2,3,5-trimethyl arabofuranose, in addition to 2,3-dimethyl xylose and a little monomethyl xylose, whereas trimethyl xylose is absent, it follows that the arabofuranose unit terminates a chain and is associated in xylan with some 16 to 17 xylopyranose units. The viscosity data and the absence of reducing power indicate marked aggregation. The authors put forward, as the simplest explanation of the facts, formula XV, and consider the vari-

ous ways in which the reducing group may be united by co-ordinate links or "hydroxyl bonds" (90) to adjoining molecules to give molecular aggregates. At present, however, the idea of a large ring of xylopyranose units with arabofuranose residues as side chains cannot definitely be excluded. The position reached is one of special interest since the possibility arises that xylan may be naturally related more closely to the plant gums than to cellulose.



XV

In conclusion, brief reference may be made to one or two other polysaccharides of biochemical interest, work on which has not yet reached a conclusive stage. These include chitin, of which a detailed x-ray examination has been made, and an arrangement is suggested for the chitobiose residues in the unit cell (91). Acetylation, nitration, and methylation of chitin have been studied, and the extreme difficulty attending each of these operations is emphasised (92). Ehrlich's view that pectins contain basic units of four galacturonic residues has been challenged and it is suggested that certain polygalacturonic acids contain about 10 such residues (93). A commencement has been made with the study of wood starch from oak and walnut and the interesting fact emerges that this substance contains some 10 per cent of uronic acid residues (which may be partly methylated) in addition to glucose residues united through α -glucosidic links (94). In contrast with this, wood from the European larch yields a polysaccharide of low molecular weight (approximately 8000) which is devoid of methoxyl and uronic acid but gives only arabinose (12 per cent) and galactose (82 per cent) on hydrolysis (95).

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UNIVERSITY OF BIRMINGHAM
BIRMINGHAM, ENGLAND

THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS*

By T. P. HILDITCH

*Department of Industrial Chemistry,
University of Liverpool*

GLYCERIDES AND THEIR CONSTITUENTS

The mixtures of mixed glycerides which constitute natural fats are built on lines which have much in common throughout wide regions of the vegetable and animal kingdoms (cf. pp. 104-106). On the other hand the mixtures of fatty acids present vary greatly, although as a rule they group themselves fairly closely with the biological relationships or classification of their sources. Most of the work to be reviewed deals, as usual, with the description of the component acids, rather than with the glyceride structure, of fats newly examined, and in considering this aspect it is convenient to subdivide them according to their origin.

COMPONENT ACIDS OF VEGETABLE FATS

In most of the following it will be found that the component acids align themselves with those of fats previously studied from other species of the same botanical family.

Fruit-coat fats.—Jamieson & McKinney (1) found that the fat expressed from fruits of the Pataua palm (*Oenocarpus pataua*) contained glycerides of oleic acid (76.5 per cent), palmitic acid (8.8 per cent), stearic acid (5.6 per cent), and linoleic acid (3.4 per cent), with a trace of triacontanoic acid. The fatty acids of "Japan wax," the glycerides present in the coat of berries of *Rhus succedanea*, are given by Tsujimoto (2) as palmitic (77 per cent), stearic and arachidic (5 per cent), oleic (12 per cent), and dibasic acids [$C_{23}H_{44}O_4$, etc. (6 per cent)].

Seed fats.—Stillman & Reed (3) found that Cuban palmiche nut oil contains the acids usually found in Palmae seed fats: decoic (5 per cent), lauric (32 per cent), myristic (16 per cent), palmitic (7.5 per cent), oleic (28.5 per cent), and linoleic (9.5 per cent). A new analysis of ucuhuba fat (*Virola surinamensis* or *V. sebifera*) by Steger & van Loon (4) gave the following distribution of acids:

* Received January 6, 1936.

lauric (12.6 per cent), myristic (63.2 per cent), palmitic (8.4 per cent), oleic (6.3 per cent), and linoleic (2.8 per cent). The same authors (5) found in coula-seed oil glycerides containing saturated acids (1.6 per cent), oleic acid (87.1 per cent), and linoleic acid (2.7 per cent); this fat is richer in triolein than any yet observed and would form an excellent source from which conveniently to prepare pure oleic acid. According to Child (6) the seed oil of *Aegle Marmelos*, used in India in dysentery, contains, like other fats of the Rutaceae, palmitic (15.6 per cent), stearic (8.3 per cent), oleic (28.7 per cent), linoleic (33.8 per cent), and linolenic (7.6 per cent), as its main component acids; the kernels contain 45 per cent of fat and about 35 per cent of protein. Some Indian acorn oils were examined by Puntambekar & Krishna (7); that of *Quercus incana* contained 17 per cent of palmitic and 82 per cent of oleic in its fatty acids.

Three further instances of seed fats in which palmitic forms about 20 per cent of the total fatty acids have been recorded. Barkenbus & Thorn (8) found that the fixed oil of rose-mallow seeds (a hybrid from *Hibiscus moscheutos* x *H. coccineus*) closely resembled cotton-seed oil—palmitic acid (15 per cent), oleic acid (33 per cent), and linoleic acid (45.5 per cent) being the main components of the glycerides. The seeds of *Pongamia pinnata* contain, according to Soliven (9), fat (26 per cent), carbohydrates (32 per cent), and protein (23 per cent); the glycerides are made up of saturated acids (23 per cent), oleic acid (11 per cent), and linoleic acid (65 per cent). Coffee-bean oil (freed from wax) was found by Schuette, Cowley & Chang (10), to include glycerides of palmitic acid (20 per cent), stearic acid (9 per cent), oleic acid (12 per cent), linoleic acid (26 per cent), and small amounts of other acids.

Lutenberg & Ivanov (11, 12) reported on the oils from ripe and immature seeds of *Datura stramonium* and *Hyoscyamus niger*. The oils from the ripe seeds had, respectively, iodine values of 126 and 143; linoleic acid formed 55 to 60 per cent of the total component acids in each oil, the remainder (apart from 12 and 7 per cent, respectively, of saturated acids) being oleic acid. Linolenic acid was not present in either.

Ito (13) studied the constituents, both fatty and unsaponifiable, of a number of Gramineae seed oils, including millet oil, from which phytosterols and a compound "miliacin," $C_{32}H_{54}O$ (0.7 per cent), were isolated in addition to the fatty acids (chiefly oleic, linoleic, and palmitic). Jamieson & McKinney (14) found the seeds of *Passiflora*

edulis to contain 18 per cent of oil suitable for edible purposes; the component acids included palmitic (6.8 per cent), stearic (1.8 per cent), oleic (19.0 per cent), linoleic (59.9 per cent), and linolenic (5.4 per cent). Täufel & Thaler (15) re-examined grapeseed-oil fatty acids very thoroughly and failed to find any indication of the presence of erucic acid, which had been reported by other workers. Jamieson & McKinney (16) reported similarly as regards the absence of erucic and myristic acids from Californian raisin oil, and gave the component acid percentages as palmitic 6, stearic 2.7, oleic 32.1, linoleic 51.9, and linolenic 2.3.

Other seed fats for which data are recorded include those of digger pine (17), *Pinus pumila* (18), fig (19), *Amoora rohituka* (20), and *Plantago ovata* (21).

Seed fats containing conjugated tri- and tetra-ene acids.—Florida tung oil, according to Jamieson & McKinney (22), has 94 per cent of eleostearic, 4.6 per cent of stearic and not more than 1 per cent of oleic glycerides, while the oil from *Aleurites trisperma* (bagilumbang) contains eleostearic acid (67 per cent), oleic acid (13 per cent), and saturated acids (17 per cent) in combination (23). Oiticica oil, from *Licania rigida* (*Couepia grandiflora*), was stated by Steger & van Loon (24) to contain an isomeric form of eleostearic acid termed couepic acid, but Brown & Farmer (25) showed that at least 30 per cent of the total acids consisted of a 4-keto-eleostearic acid (licanic acid), $\text{CH}_3(\text{CH}_2)_3 \cdot (\text{CH}:\text{CH})_3 \cdot (\text{CH}_2)_4 \cdot \text{CO} \cdot (\text{CH}_2)_2 \cdot \text{COOH}$. According to Kappelmeier (26) eleostearic and licanic acids may both be present. The seed fat of *Parinarium laurinum* had also been reported (27) to contain an isomer of eleostearic acid which Farmer & Sunderland (28) have now shown to be in reality a tetraene acid, $\text{CH}_3 \cdot \text{CH}_2 \cdot (\text{CH}:\text{CH})_4 \cdot (\text{CH}_2)_7 \cdot \text{COOH}$; this is apparently the first recorded occurrence of a tetraene acid in a land-vegetable seed fat, although such acids occur in small amounts in many land-animal fats (arachidonic acid) and are common in fats of aquatic origin. Brown & Farmer (29) confirmed that *P. macrophyllum* seeds contained α -eleostearic acid, as already reported by Steger & van Loon (30), and this acid was also shown by Farmer & Paice (31) to be present in quantity in the seed fat of *Telfairia occidentalis*. [It is therefore possible that "telfairic acid," reported by Thoms (32) in *T. pedata* as an isomer of linoleic acid, may also be eleostearic acid.]

The occurrence of the unusual eleostearic acid in seed fats of isolated species of three different families, Euphorbiaceae, Rosaceae,

and Cucurbitaceae, forms a striking exception to the usual rule that specific seed fatty acids (especially those other than palmitic, oleic, and linoleic) tend to be restricted to members of one, or at the most a few, plant families. Meanwhile, other isomeric forms of eleostearic acid (punicic and trichosanin acids) have been reported by Toyama & Tsuchiya (33, 34) in the seed fats of pomegranate and of *Trichosanthes cucumeroides* respectively.

COMPONENT ACIDS OF ANIMAL-DEPOT FATS

Schuette, Garvin & Schwoegler (35) found the component acids of abdominal fat from a Western range horse to contain palmitic acid (26.3 per cent), stearic acid (4.5 per cent), oleic acid (46.8 per cent), linoleic acid (11.9 per cent), and linolenic acid (4.5 per cent). Morrison (36) described the perinephric fat of the Australian emu [m.p. 30 to 31°, iodine value (Wijs) 95.5], which was made up of 40 per cent saturated (palmitic and stearic), 49 per cent oleic, and 11 per cent linoleic acid. Klenk, Ditt & Diebold (37) determined the percentage composition of the depot-fat acids of the rat, lizard (*Varanus salvator*), and Greek tortoise; the results are presented in the following table:

TABLE I
PERCENTAGE COMPOSITION OF DEPOT-FAT ACIDS

	Saturated			Unsaturated		
	C ₁₄	C ₁₆	C ₁₈	C ₁₆	C ₁₈	C ₂₀₋₂₂
Rat	2	25	3.5	13	55	1.5
Lizard	4	18	7	10	56	5
Tortoise	1	14	4	9	65	7

The unsaturated C₁₆ acids were monoethenoid and the C₁₈ acids had a mean unsaturation of -2.4 to -2.6 H in each case, but that of the C₂₀₋₂₂ acids was -2.9 H in the rat, -3.2 to -5.3 H in the tortoise, and -3.6 to -5.5 H in the lizard. The content of C₂₀₋₂₂ acids and their mean unsaturation thus appeared to be dependent on the body temperature.

GLYCERIDE STRUCTURE OF DEPOT FATS

A review of recent work on this subject was given by Hilditch (38). It may be useful here to sum up the present position with references to the relevant literature. In most natural fats the glyceride composition tends toward that in which the different fatty acids are

distributed as evenly as possible in combination throughout all the triglyceride molecules. This rule is followed extremely closely in practically all seed fats which have been examined. Hilditch, Collin & Lea (39, 40, 41, 42) observed this by quantitative study of the content of triglycerides containing three saturated acyl residues in several solid seed fats, and subsequent work on a wide variety of other solid seed fats (43, 44, 45, 46, 47) confirmed it. That the more unsaturated or liquid varieties of seed fats possess a similar general type of "mixed glyceride" structure was later shown by Hilditch & Jones (48) from a study of the hydrogenated products from several liquid oils. The somewhat earlier work of Suzuki and others (49, 50, 51), who submitted the crystalline bromo-addition products of linseed, soy-bean, whale, cod-liver and other fish oils to qualitative separation, indicated the presence of many mixed glycerides in both the seed oils and the marine animal oils examined. In a number of fruit-coat fats (e.g. palm and olive oils), on the other hand, there has been found a minor tendency toward the occurrence of more tripalmitin than is consistent with "even distribution" of fatty acids in the triglyceride molecules to the extent to which this is seen in seed fats (42, 52); but for the most part fruit-coat glycerides are still mainly of the "mixed" type (47, 53, 54).

In animal-depot fats which contain little stearic acid in addition to the usual 25 to 30 per cent of combined palmitic acid [for instance in the rat (55)] the "evenly distributed" type of glyceride structure is also found. But the depot fats of many domestic animals contain important proportions of combined stearic acid as well as palmitic acid and in these (and also in the milk fats) the content of fully-saturated glycerides is much greater than that observed in, for example, seed fats containing the same ratio of saturated to unsaturated acids (56, 57, 58, 59). The abnormal amounts of fully-saturated components, coupled with the fact that in the depot fats the combined content of stearic and oleic acids is not far from constant in spite of fairly wide variations in the amount of either acid, have led to the suggestion that the stearo-glycerides of the depot fats in question, and the characteristic lower saturated glycerides of the milk fats, may have been produced from preformed, "evenly distributed" mixed oleo-glycerides. Hilditch & Stainsby (59) found recently that a soft depot fat of the pig, when hydrogenated in successive stages, progressively increased in fully-saturated glyceride content in a manner which simulated closely the behaviour of a number of pig, ox, and sheep depot

fats and cow or buffalo milk fats. The technique of progressive hydrogenation also led to a more quantitative statement than previously of the component glycerides originally present. The pig fat contained only 15 per cent of tri- C_{18} -glycerides (? steardiolein); nearly 80 per cent consisted of a mixture of monopalmito-diunsaturated glycerides with palmitostearo-oleins, and much, if not all, of the palmitic acid was attached to the β -hydroxyl group of the glycerol molecule.

The same workers (60) found that in hen-body fats (which are not unlike pig fat in component acids, except for the presence of 8 per cent of palmitoleic acid) the glycerides were differently composed, and included about 30 per cent each of tri- C_{18} - and of dipalmito- C_{18} -glycerides, with only 40 per cent of monopalmito-glycerides. The examination of fats after progressive hydrogenation to various stages as well as to complete saturation also has been applied to the cases of palm oils (61), piquia fat (54), and rape oil (62). The relative increase in fully-saturated components during progressive hydrogenation of fats which differ in their original content of saturated fatty acids was discussed by Hilditch & Paul (62).

Verkade & van der Lee (63) outlined general methods for the synthesis of mixed triglycerides of known configuration by means of triphenylmethylglyceryl esters, with special reference to possible migration of acyl groups from β - to α -positions during hydrolysis of the triphenylmethyl derivatives.

COMPONENT ACIDS OF LIVER GLYCERIDES AND PHOSPHATIDES

Klenk (64) analysed the liver glycerides and liver phosphatides of the Greek tortoise (*Testudo graeco*); when tabulated with his previous data for the corresponding components of the livers of ox (65) and frog (66), and of their depot fats, interesting parallels are to be observed (cf. Table II, p. 107).

The liver glycerides are not greatly dissimilar from the corresponding depot fats, but the phosphatides contain much less C_{18} unsaturated acids, and frequently also less palmitoleic acid, while the C_{20-22} acids are much increased. The mean unsaturation of the latter group is also much higher, and that of the C_{18} group is increased in the phosphatides as compared with the glycerides. It becomes increasingly clear that the relatively high average unsaturation (or iodine value) of liver fats is concentrated in the phosphatide fraction and that here it is due to the increased proportion (and unsaturation) of the

C₂₀₋₂₂ acids and not to any increase in quantity or unsaturation of the C₁₈ acids. Klenk has pointed out the obvious inadequacy of the simple hypothesis of desaturation of fatty acids in the liver to explain these facts. A further moral should also be drawn, namely, the extreme undesirability of placing any great weight, or elaborating any broad hypotheses, upon observations of the iodine value or mean unsaturation of any natural fat as such, without also resolving it into, at least, its main component acids.

TABLE II
COMPONENT ACIDS OF GLYCERIDES AND PHOSPHATIDES IN LIVER AND
DEPOT FAT
(Percentage distribution)

	Saturated Acids			Unsaturated Acids*		
	C ₁₄	C ₁₆	C ₁₈	C ₁₆	C ₁₈	C ₂₀₋₂₂
Ox:						
Depot glycerides....	6	27	21	46(2.2)
Liver glycerides....	..	25	20	9(2.0)	37(2.6)	9(5.0-7.4)
Liver phosphatides..	..	12.5	27	5	27(2.6-3.4)	28.5(5.3-8.2)
Tortoise:						
Depot glycerides....	1	14	4	9(2.0)	65(2.4-2.6)	7(3.2-5.3)
Liver glycerides....	1	11	4	15(2.0)	66(2.1)	3(2.7-4.5)
Liver phosphatides..	..	15	10	10(2.0)	48(2.8)	17(5.7-6.5)
Frog:						
Depot glycerides....	4	11	3	15(2.0)	52(ca.3)	15(ca.6)
Liver glycerides....	..	21	..	← 61(ca.3) →		18(ca.6)
Liver phosphatides..	..	25	42(ca.3)	33(ca.6)

* Figures in parentheses refer to the mean unsaturation; e.g., "2.0" signifies "—2.0 H," i.e., monoethenoid.

Irving & Smith (67) have analysed the mixed fatty acids obtained by saponification of pig livers. Tsujimoto & Koyanagi (68) examined the liver of a whale shark (*Rhinodon typicus*) which yielded 55 per cent of semi-solid fat (iodine value 108.6 and unsaponifiable matter 5 per cent). The unsaponifiable fraction contained 44 per cent of cholesterol and also selachyl and probably batyl alcohol; the fatty acids were of the usual marine-oil type, with very high unsaturation in the C₂₀ and C₂₂ group.

Sinclair (69) has shown that the ratio of saturated to unsaturated acids in the phosphatides of the liver, skeletal muscle, and kidney of the rat is constant, irrespective of the degree of unsaturation

of the mixed acids, and has devised a micromethod by which the proportions and iodine values of solid and liquid acids may be determined in 30 to 40 mg. of mixed fatty acids. Feeding of a fully-saturated fat, or prolonged fasting, has no effect on the high degree of unsaturation of these tissue phosphatides induced by daily feeding of a small amount of highly unsaturated fat.

COMPONENT ACIDS OF FATS FROM AQUATIC FLORA AND FAUNA

Constitution of unsaturated acids of marine fats.—Toyama & Ishikawa (70) showed that the acid, $C_{20}H_{38}O_2$, present in the blubber fat of northern rorqual and humpbacked whales is the same (9-eicosenoic acid, gadoleic acid) as that from cod-liver, herring, and other oils, for which Toyama & Tsuchiya (71) had proved the structure as stated; pilot-whale oil was shown to contain an isomeric acid, gondoic acid, which differs from gadoleic acid in the position of the ethenoid linking. Toyama & Tsuchiya (72) reported that herring, cod-liver, pilot-whale, and some shark-liver oils contain small amounts of highly unsaturated C_{24} acids. The most unsaturated portion corresponded with the formula $C_{24}H_{36}O_2$ (nisinic acid) and thus contained the equivalent of six ethylenic linkings; on hydrogenation it gave *n*-tetracosanoic acid.

The highly unsaturated acids of Japanese sardine oil have received much attention. Clupanodonic acid, $C_{22}H_{34}O_2$, was submitted to ozonolysis by Toyama & Tsuchiya (73) who suggested the structure 4,8,12,15,19-docosapentaenoic acid, differing only in detail from the conclusions arrived at previously by similar means by Tsujimoto (74), namely, that the acid was either 4,7,11,15,19- or 4,8,11,15,19-docosapentaenoic acid. On the other hand Inoue & Kato (75) compared the similar behaviour toward potassium iodide of the decabromide of clupanodonic acid and the tetrabromide of stearolic acid, and considered that this supported the view that an acetylenic linking was present in clupanodonic acid; Inoue & Sahashi (76) had previously suggested the structure 4,7,11-docosatrien-18-inic acid as a result of oxidations of various partially hydrogenated clupanodonic acids. Kino (77), however, following ozonolysis of clupanodonic esters which had been partially polymerised by heat, was unable to reconcile his results with the presence of an acetylenic group in clupanodonic acid, although the structure of the partially polymerised products could be explained according to constitutions such as those suggested by Tsujimoto or by Toyama & Tsuchiya.

Toyama & Tsuchiya (78) separated other highly unsaturated acids from Japanese sardine oil and submitted their methyl, ethyl, or amyl esters to ozonolysis. They suggested constitutional formulae as follows: $C_{16}H_{26}O_2$, hiragonic acid, 6,10,14-hexadecatrienoic acid; $C_{18}H_{28}O_2$, moroticic acid, 4,8,12,15-octadecatetraenoic acid; $C_{20}H_{32}O_2$, 4,8,12,16-eicosatetraenoic acid; $C_{20}H_{30}O_2$, 4,8,12,15,18-eicosapentaenoic acid; $C_{22}H_{32}O_2$, 4,8,11,14,17,20- or 4,8,12,15,18,21-docosahexenoic acid; $C_{24}H_{36}O_2$, 4,8,12,15,18,21-tetracosahexenoic acid.

It seems likely that the structures of some of these polyethenoid acids still remain somewhat uncertain. The mixture of acids yielded by their oxidation is formidably complex, and individual identification is an extremely difficult matter.

Fish oils.—Tsujimoto & Koyanagi (79) continued their study of fats of shell fish. The tropical "shako," a very large shell fish, yielded fat with little phosphatides; the fatty acids had an iodine value of 91 and gave 16 per cent of ether-insoluble bromides; the unsaponifiable matter included a new isomeride of cholesterol, "shakosterol" (acetate, m.p. 156°). The small, conical "kumanokogai" contained both phosphatides and fats, the sterol present being chiefly cholesterol; the fatty acids had an iodine value of 153 and gave 40 per cent of ether-insoluble bromides. Tsujimoto (80) found the flesh of *Squalus sucklii* contained a highly unsaturated fat (acids, iodine value 178 to 187; ether-insoluble bromides 54 to 63 per cent); most of the acids were of the C_{18} , C_{20} , or C_{22} series and the unsaponifiable fraction (5 to 8 per cent) included batyl and selachyl alcohols. Ueno & Komori (81) examined Itoyo fish oil (from *Gasterostenus aculeatus*), the acids from which were of the usual "marine" type, rich in palmitoleic, oleic, moroticic, and clupanodonic acids; the unsaponifiable matter (1 per cent) contained, as well as cholesterol, oleyl alcohol, and a higher polyethenoid alcohol.

Lovern (82) made detailed analyses of salmon-ova fat, and of salmon-depot fat at various periods of the life cycle. The ova fat is more unsaturated than the depot fat of the spawning female; the young salmon living in fresh water develop fat of the fresh-water fish type, and on passing to the sea the depot fat is transitional in composition between that of the young and the mature salmon. Progressive changes in the depot fat of salmon which had returned to the rivers for spawning and which were then not feeding were also recorded in detail. Lovern (83) examined the component acids in the fats of four plankton Crustacea, and (84) compared the body fat of

grass-feeding carp with those of other fresh-water fish and with the glycerides present in the grass feed. In all these his conclusions were based on the fatty acid compositions revealed by ester-fractionation analyses.

INDIVIDUAL FATTY ACIDS

In many cases the structure or properties of individual acids have already been dealt with in previous pages under the fats of which they are components. A few references to oleic and linoleic acids remain to be mentioned. Täufel & Künkele (85) described useful methods for the preparation of oleic acid chloride and of α -mono-olein. The chloride is best made from the acid and phosphorus trichloride; use of the pentachloride or of thionyl chloride leads to the formation of undesired by-products.

Toyama & Tsuchiya (86) found that thiocyanogen unites with linoleic and with linolenic acids respectively in the 9,10- and in the 9,10,12,13-positions, whereas, during incomplete addition of bromine, the positions attacked are respectively the 12,13 in linoleic, and the 12,13,15,16 in linolenic acid. With thiocyanogen, therefore, addition commences preferentially at the double bond nearest the carboxyl group, while with bromine the reverse holds. Green & Hilditch (87) reviewed methods for the identification of linoleic and linolenic acids but found no improvement on the alkaline oxidation of the acids to tetra- or hexa-hydroxystearic acids (Hazura), although the yields by this method are not satisfactory. They (87) studied further the C_{18} unsaturated acids in butter fat and concluded that an octadecadienoic (but not, as a rule, ordinary linoleic) acid is present therein in some quantity [cf. Bosworth & Brown (88), Eckstein (89)].

CONSTITUENTS OF WAXES¹

Piper, Chibnall & Williams (90) gave comprehensive data for the melting points and X-ray spacings of normal alcohols, acids, and derivatives containing from 26 to 36 carbon atoms in the molecule and for binary and ternary mixtures of known composition, and indicated the mode of use of the data in studying the corresponding components of natural waxes. Chibnall, Piper, Pollard, Williams & Sahai (91) gave a full account of their valuable studies by these methods of the mixed alcohols (even, C_{24} to C_{36}), acids (even, C_{24} to C_{34}) and paraffins (odd, C_{25} to C_{37}) in many plant and insect waxes and pro-

¹ Cf. also this volume, p. 238. (EDITOR.)

posed important revisions in the nomenclature of a number of the higher alcohols and acids. Chibnall & Piper (92) suggested a general scheme for the metabolism of natural waxes based on the hypotheses that (a) primary alcohols result from reduction of the corresponding acids, (b) paraffins, secondary alcohols, and ketones from decarboxylation of acids containing one carbon atom more than the derivatives in question, and (c) saturated, keto, or hydroxy acids arise from unsaturated acids. Mattissohn (93) confirmed that the so-called cerotic acid of beeswax is a mixture of 20 per cent of *n*-tetracosanoic acid with higher homologues.

Collins (94) found the acids of Chinese grass wax to be mixtures of saturated C_{24} , C_{26} , C_{28} , and C_{30} acids, while those of Esparto grass wax included C_{32} and C_{34} acids and were somewhat higher in molecular weight; the hydrocarbons in Esparto and Candelilla waxes were chiefly hentriacontane, with about 4 per cent of other hydrocarbons. Markley, Hendricks & Sando (95) found the wax-like coating of the pear to contain ursolic acid, fatty acids (predominantly oleic, with little linoleic or linolenic, and about 12 per cent of saturated C_{14} to C_{24} acids), primary alcohols from C_{20} to C_{30} , and *n*-nonacosane. Blount (96) observed in the insect wax of felted beech coccus a mixture of primary alcohols and acids (about 80 per cent of C_{26} with C_{24} and C_{28} in each case).

HIGHER ALCOHOLS AND HYDROCARBONS

In addition to the above the following alcohols have been reported. André & Bloch (97), who stated (98) that batyl and selachyl alcohols are present in marine oils in the form of esters, $R'O \cdot C_8H_5(CO_2 \cdot R'')$ ($CO_2 \cdot R'''$), have given the composition of a liver oil of *Scymnorrhinus lichia* as squalene 57 per cent, triglycerides 21.7 per cent, and mixed ether-esters of batyl, etc., alcohols 21.3 per cent; the latter are highly laevorotatory. Ueno & Yamasaki (99) isolated from the unsaponifiable matter (8.6 per cent) of the corn of *Andropogon Sorghum vulgaris*, in addition to much sitosterol, "koryanyl alcohol" $C_{28}H_{58}O$, and "takakibyl alcohol" $C_{44}H_{90}O$.

Thorbjarnarson & Drummond (100) found that the highly unsaturated, unsaponifiable matter from Palestine, Tunisian, Spanish, and Turkish olive oil contains, respectively, about 64, 38, 31, and 58 per cent of squalene, which was not detected in tea-seed oil. Tsujimoto (101) observed that the liver oil of the basking shark, *Cetorhinus maximus*, contains squalene, pristane (*iso*-octadecane, 6 per cent),

and "zamene" (an octadecylene, $C_{18}H_{36}$). Toyama & Tsuchiya (102) stated that pristane occurs in small amounts in sardine, herring, sperm, and other oils. Nakamiya (103) isolated another unsaturated hydrocarbon ("gadusene," $C_{18}H_{32}$) from the unsaponifiable fractions of rice-germ, soya and some fish-liver oils; it had an absorption spectrum similar to, and possibly the same as, the hydrocarbon $C_{18}H_{32}$ obtained from wheat-germ oil by Drummond, Singer & MacWalter (104), and from Ishinagi-liver oil by Tsujimoto (105). Channon, Devine & Loach (106) found in pig livers an unsaturated hydrocarbon, $C_{45}H_{76}$ or $C_{50}H_{84}$ (29 mg. per 100 gm. of liver), with smaller amounts of a saturated and of a second unsaturated hydrocarbon.

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DEPARTMENT OF INDUSTRIAL CHEMISTRY
UNIVERSITY OF LIVERPOOL
LIVERPOOL, ENGLAND

THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS*¹

BY CLAUDE RIMINGTON

*Onderstepoort Veterinary Research Laboratory,
Pretoria, South Africa*

Since the reviews in previous years have dealt more particularly with the physical chemistry of the proteins and amino acids and with problems of their metabolic transformations, the writer of this present review has attempted to stress more the purely chemical aspect of the subject. The material has been approached from the point of view of an organic chemist interested in problems of structure and the manner in which structural peculiarities obtrude themselves in physiological processes.

REVIEWS

Certain reviews of protein and amino acid chemistry not already mentioned in the *Annual Review* have appeared during 1933 to 1935. Among these is an article by Linderstrøm-Lang (1), dealing with the application of enzyme preparations in the elucidation of protein structure and revealing a very valuable weapon of constitutional attack, which is being forged by the systematic synthesis of peptides of known composition and the use of these substances to define the hydrolytic activities of carefully purified enzyme preparations. Once the potentialities of these destructive agents are known, they may be used in various combinations to bring about step-wise degradation of any particular protein.

The chemistry of the serum proteins was subjected to a critical review by Rimington (2) in the same year and due stress was laid upon Sørensen's conception of soluble proteins as mutually interacting component systems. The exact nature of the state of affairs existing in normal serum is still very imperfectly understood; it is even questionable whether one can logically speak of serum albumins and globulins. A further discussion of this subject in the light of developments which have taken place during the present year will be found below.

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¹ The editor regrets that the exigencies of space have required the omission of several sections (sulphur-containing amino acids, hemoglobins, immunology, x-ray studies) together with a number of informative tables.

Emphasis is again laid by Bonot (3) upon proteins as a mixture of homeomeres in a review which seeks to interpret many physical properties of proteins from this standpoint.

The zwitterion theory of the structure of amino acids, polypeptides, and proteins, and problems arising out of this conception are treated in two reviews by Ashmarin (4) and Sadikov (5), respectively, while Roche (6) has contributed a review upon the molecular weights of proteins.

A systematic development of Gibbs' fundamental equations to protein systems by Adair (7) is of such general importance to warrant citation as a review of the theory of membrane equilibria and its extensions to systems of many components. Among other topics, the determination of molecular weights, hydration, reactions that do not obey the law of constant proportions, and the equilibrium of a protein in gravitational and centrifugal fields receive adequate treatment.

NEWLY INVESTIGATED PROTEINS AND AMINO ACIDS

Csonka (8) has studied the proteins obtainable from bakers' and brewers' yeast, *Saccharomyces cerevisiae*, and reports figures for their content in cystine, tryptophane, tyrosine, arginine, histidine, and lysine. An observation is recorded for which no adequate explanation is as yet forthcoming, namely that hydrolysing yeast directly by acid occasions a large destruction of histidine and cystine. These amino acids do not belong to that class known to play a large part in "humin" formation when carbohydrate is present and their destruction is therefore somewhat surprising and would seem to invite further study.

A new method for obtaining proteins from organs or tissues, the so-called "histoclastic" technique in which a paste of the material in water is subjected to high pressure at a low temperature has been applied by Achard & Piettre (9) in the case of liver cells. The extract contained amino acids and a phosphoprotein of the globulin type but no serum proteins. As a method of obtaining cell proteins in the original state, unaltered by chemical agencies, the method would seem promising but of course it could only be expected to yield those proteins which are soluble and not part of fixed structures.

In a study of the proteins of mulberry leaves and their availability for the growing silkworm, Kishi (10) has shown that the main protein fraction, which is soluble in alkaline alcohol, increases with respect to total protein as the leaf matures. Young leaves were most

easily digested by the worms and a relation was shown to exist between the quantity of silk protein produced and the quantity and quality of protein and carbohydrate in the leaves. The article summarises the experimental findings with regard to season, age, etc., and protein content of the leaf.

Sharpenak *et al.* (11) have taken advantage of material provided by the amputation of a leg of a boy aged eight and one-half years to report the amino acid analysis of human muscle proteins. It would appear that the whole muscle was used. The figures in Table I are given as grams per 100 gm. of muscle.

TABLE I
AMINO-ACID CONTENT OF HUMAN MUSCLE

Glycine + alanine.....	4.92	Phenylalanine	5.25
Valine	6.44	Tyrosine	4.11
Leucine	8.88	Tryptophane	2.3
Arginine	8.81	Cystine	2.27
Histidine	2.39	Proline	4.16
Lysine	6.57	Ammonia	1.24

The proteins of wheat flour have been investigated by Krejci & Svedberg (12, 13) using the ultracentrifuge. Gliadin was found to be stable in alcohol even at pH 1.46 but at alkalinities greater than pH 12 the sedimentation constant dropped. The preparations were not, however, homogeneous at pH 2.23 or greater and 20° or lower, there being a mixture of whole and half molecules with weights of 34,500 and 17,250 respectively, but by raising the temperature or increasing the acidity, dissociation to half molecules could be made approximately complete. The dissymmetry numbers were 1.92 and 1.21 for whole and half molecules, respectively.

Haugaard & Johnson, it will be remembered, showed that alcoholic solutions of gliadin could be fractionated by suitable manipulation into components of differing solubility, optical activity, etc. Utilising the same technique, Krejci & Svedberg (12) obtained a most-soluble fraction consisting almost entirely of the lower molecular weight constituent and a least-soluble fraction with a relatively high concentration of the heavier molecules. Using potassium halides in 0.5 *N* solution as extractants for proteins from wheat flour it was found (13) that proteins of increasing average molecular weight were extracted in the order KF, KCl, KBr, KI. Successive extracts were made by 0.5 *N* KF and KCl and the solutions dialysed. A hetero-

geneous mother liquid containing low molecular weight constituents remained, while the precipitate consisted largely of gliadin and wheat globulin. The latter (sedimentation constant, 11) polymerised in concentrated solution but dissociated again on dilution.

Miller (213), in a recent study, reports the arginine, histidine, and lysine contents of proteins isolated from several pasture grasses. Comparison of the Block, Vickery, Hausmann and Van Slyke methods was made and seriously high values for total basic nitrogen in the latter were shown to be due to the precipitation occurring in hydrochloric acid solution instead of sulphuric.

A new phosphoprotein, serum vitelline, has been described by Laskowski (14). It is present only in the serum of laying hens [Laskowski (15); Roepke & Hughes (16)] and the inference is that it is physiologically related to the phosphoproteins of the egg. It contains 0.95 per cent of phosphorus.

Stanley (17) reports the isolation of a non-dialysable crystalline protein possessing the properties of tobacco-mosaic virus. The molecular weight would appear to be very high and it is stated that the loss of activity occurring on peptic digestion or coagulation is proportional to the native protein disappearing. The proof that a material of such complexity is really an active agent and not an association of the latter with an inert carrier is always a matter of great difficulty. Should the present claims be substantiated, however, fresh light may be thrown upon the vexing question of the vital or non-living character of viruses.

The crystallisation of chymotrypsin and of chymotrypsinogen, its precursor, a new proteolytic enzyme from the pancreas, adds one more to the list of enzymes which have now been obtained in the crystalline state. The investigation by Kunitz & Northrop (18) is very thorough. Chymotrypsinogen is activated by minute amounts of trypsin but not by enterokinase, pepsin, calcium chloride, or inactivated trypsin. The course of activation is monomolecular with a maximum at pH 7.0 to 8.0, and 6 amino groups per molecule make their appearance without, however, any split-products being formed. The change in optical rotation is slight and any change in molecular weight undetectable, facts all of which indicate that activation must be associated with some internal rearrangement. Chymotrypsin hydrolyses sturin, casein, gelatin, and hemoglobin, attacking different linkages from those opened up by trypsin. It has no action upon di- or polypeptides.

The peptide nature of insulin is firmly established and Jensen & Evans (19) by the use of phenylisocyanate and α -naphthyl isocyanate now show that a part, at least, of the free amino nitrogen is due to phenylalanine. Treatment of crystalline preparations with the isocyanates reduced the activity by about 95 per cent, no ammonia being evolved when sodium hydroxide was subsequently added. The ammonia-liberating groups are therefore affected by isocyanate. Hydrolysis gave proline and phenylalanine as the phenylhydantoin. Practically the same yields of these amino acids were obtained when alkali-treated insulin was used as the starting point, indicating the complexity of the reaction between insulin and sodium hydroxide whereby, as is well known, one sign of change is the liberation of ammonia.

The suggestion has recently arisen that another hormone, the oxytocic principle of the posterior lobe of the pituitary, is a peptide or is very closely associated with a peptide. Freudenberg *et al.* (20, 21) show that it constitutes a reduction-oxidation system in which sulphur probably plays a part, yields tyrosine, cystine, and small amounts of histidine after hydrolysis, and is easily inactivated by proteolytic enzymes, hydrogen peroxide, alkaline iodine solutions, and comparable agents. The total sulphur is 3.2 per cent.

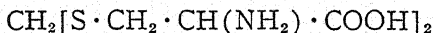
Against these views must be ranged the experiments of Gulland *et al.* (22) who, by approaching the problem from the physical angle, conclude that the ultraviolet absorption spectra of different preparations correspond to peptone-like impurities but not to the hormone itself. Electrodialysis effected a degree of separation but the stability of the hormone was impaired. At pH's greater than 8 it remained in the centre of the cell but migrated to the cathode below pH 6. They report that it is inactivated by acetic anhydride but no regeneration of activity occurred at pH 10 in the course of half an hour at the temperature of the testing bath. These observations, together with that of Gulland (23) that the hormone is susceptible to attack by nitrous acid, would indicate that it is probably a base rather than an ampholytic peptide.

It is considered to be of sufficient general interest to refer to the isolation by Jacobs & Craig (24) of the amino acids *l*-phenylalanine and *d*-proline from the non-lysergic-acid portion of ergotinine, one of the ergot alkaloids. They point out that combination of lysergic acid, proline, phenylalanine, ammonia, and isobutyrylformic acid (recognised as a constituent part of the molecule) with loss of 4 molecules of water would quantitatively constitute ergotinine. Alkaloids

derived from simple amino acids are not numerous and the discovery that two, at least, enter into the composition of this remarkable product of the ergot mycelium is especially noteworthy. It will be noticed that *d*- and not *l*-proline was isolated.

Another outstanding contribution which should command general interest is that of Dakin & West (25) who have studied a purified hematopoietic substance obtained by them from liver. It appears to be a glyco-peptide and yields, on hydrolysis, an amino hexose similar to glucosamine, lysine, arginine, glycine, leucine, hydroxyproline, and aspartic acid. Histidine, glutamic acid, and possibly traces of phenylalanine were also found in small quantities in certain preparations but pyrimidine and purine bases were absent. It is slowly and slightly digested by pepsin, completely by erepsin. The material is not yet pure but sufficient has been done to make us await further developments with the keenest interest. In particular, since the liver antianemic substance is known to be derived from ingested protein, a comparison with carbohydrate groups known to occur in proteins (see discussion below) should prove to be illuminating.

A new sulphur-containing amino acid, "djenkolic acid" has been isolated by van Veen & Hyman (26) by hydrolysing djenkol nuts (*Pithecolobium labatum*) with baryta at 30°. Its composition is given as $C_7H_{14}N_2S_2O_4$ (m.p. about 250°; $[\alpha]_D^{20} = -25^\circ$ for a 2 per cent solution in 1 per cent HCl). The dibenzoyl derivative melts at 85°. Djenkolic acid is not changed by hot concentrated hydrochloric acid and saturated baryta gives neither sulphite nor sulphate. Hydrolysis by concentrated sulphuric acid, however, yielded cystine and formaldehyde. It is not reduced by zinc and dilute acid. With potassium cyanate it gives a dihydantoin (m.p. 200°) which loses sulphur easily on warming with diluted acids or alkalis. On the basis of these reactions the following structural formula is proposed:



β -hydroxyglutamic acid was first isolated by Dakin but in several instances subsequent workers have found it difficult to obtain this amino acid from protein hydrolysates. Gulland & Morris (27) now bring forward an improved method of isolation involving adsorption on barium sulphate, electrodialysis, and phosphorylation. They record a yield of 0.33 per cent from casein. It will be remembered that Dakin (28) obtained yields of about 10 per cent from casein but Harington & Randall (29), who effected a new synthesis of the in-

active amino acid, reported complete failure to obtain any at all from natural sources by following Dakin's procedure. Rimington (30) obtained it from his phosphopeptone of casein after complete hydrolysis.

The imidazole derivatives, carnosine and anserine,² have now been found in mammalian skeletal muscle by Wolff & Wilson (31), experimental material being obtained from the dog, cat, deer, gnu, opossum, and llama.

Careful arginine determinations by the newer methods now available lead Fuchs (32) to deny the existence of any guanidine derivative other than arginine in pseudomucin, thus disposing of the earlier suggestions of Otori (33) based on less reliable determinations.

METHODS

The search for specific precipitants for the various amino acids continues. Bergmann & Fox (34) report experiments upon the complex formed between glycine and potassium trioxalatocobaltate and illustrate its use by the determination of glycine in a gelatin hydrolysate. A colorimetric method for glycine determination, due to Patton (35), is carried out by adding benzaldehyde to the hydrolysis mixture immediately after the protein has dissolved. Tryptophane is thereby destroyed and the colorimetric procedure which follows is an adaptation of Klein & Linser's (36) reaction using σ -phthalic aldehyde. Results with various proteins are listed. The sum of glycine and serine together may be determined according to Rapoport's (214) method depending upon the permanganate oxidation of derived glycolic and glyceric acids to oxalic acid.

The identification of small quantities (order of 1 mg.) of glycine, leucine, phenylalanine, asparagine, and valine is the subject of a study by Fosse, de Graeve & Thomas (37). The substance is converted into its hydantoic acid and estimated as the xanthylhydantoate. A colour reaction between isatin in acetic acid solution and pyrrolidine, proline, or hydroxyproline is described by Grassmann & Arnim (38). Only those proline peptides with terminal proline nitrogen react and it would appear that in these cases the amino acid is first set free by hydrolysis.

Greenbaum (39) describes the separation of large amounts of tyrosine from cystine.

A new method for the determination of cysteine and cystine in

² Cf. *Ann. Rev. Biochem.*, 2, 370 (1933).

proteins is described by Rossouw & Wilken-Jorden (40). This should be particularly welcome since it embodies several entirely new features and appears to be free from the disadvantages attached to the methods now in use, all of which follow much the same procedure for the separation of cystine from the hydrolysate with consequent unavoidable losses. In this method cystine is removed as the cysteine cuprous mercaptide [compare Vickery & White (41)] and is estimated colorimetrically by means of the modified Sullivan method as described by Rossouw & Wilken-Jorden (42). This method would appear to be the most reliable so far proposed for cystine determination. Hess & Sullivan (43) adduce evidence to show that the Sullivan colorimetric technique is applicable to the determination of cystine partitioned between butyl alcohol and *N* hydrochloric acid.

Iodometric titration of cysteine, by the indirect back-titration method, is shown by Lavine (44) to be possible in a solution molar in hydrochloric acid and potassium iodide. The reaction between thiol compounds and mercuric chloride at pH 5 has been investigated by Shinohara (215) and shown to take place in two stages. Two papers are also devoted by Shinohara (45, 46) to a critical study of the reaction between thiol compounds and the phospho-18-tungstic acid reagent and the interference caused by other substances. It is clear that our knowledge of these complex reactions is far from complete and much is still empirical. A scheme for the determination of thiol compounds is, however, finally worked out by the author.

The optimal conditions for the precipitation of *l*-, *dl*-, and *m*-cystine by phospho-12-tungstic acid have been worked out by Toennies & Elliott (47). In *N* HCl, *m*-cystine can be precipitated to a residual concentration of 5×10^{-4} *M*. The crystalline precipitates vary in composition according to the ratios of the components used.

Mirsky & Anson (48) propose methods for the determination of -SH and -SS- in proteins based upon interaction between the protein-sulphur group and an added thiol compound. Thus, protein -SH may be determined by the amount of added cystine it reduces (also as cysteine after complete hydrolysis). -SS- groups are determined by the increase in -SH after treatment of the protein with thioglycolic acid.

Colorimetric determination of cysteine in a protein hydrolysate is possible by the use of the Folin-Marenzi reagent without the addition of sulphite but a disturbing factor in colour intensity is the amount of alkali present [compare Rimington (49)].

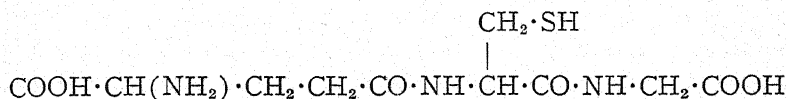
Tryptophane determination by means of the colour produced with Ehrlich's reagent and using reduced phosphomolybdate as a comparison standard is the method recommended by Tomiyama & Shigematsu (50). One disadvantage is that colour development must take place at a strictly controlled temperature of 30° for six to seven days. The figures obtained with several proteins are recorded.

Fine (51) describes an adaptation of the biuret method to the determination of albumin and globulin in serum and urine, while an ultrafiltration followed by determination of acid-binding power forms the basis of a method proposed by Freudenberg (52) for the determination of proteins in body fluids. The values so obtained are higher than those yielded by the Kjeldahl method.

Nadeau & Branchen (53) have shown that amino acids dissolved in glacial acetic acid act as sufficiently strong bases to be titrated with 0.1 *N* perchloric acid to a high degree of accuracy, determining end-points potentiometrically or by means of indicators such as crystal violet, α -naphtholbenzein or benzoyl auramine.

SYNTHESES AND PREPARATIONS

Glutathione has been synthesised by Harington & Mead (54) thus placing its constitution beyond all doubt. The tripeptide, γ -glutamyl-cysteylglycine, has the following formula :



The difficulty of applying the carbobenzoxy method to the synthesis of a sulphur-containing substance such as glutathione was overcome by introducing a reduction with phosphonium iodide at 45 to 50° in acetic acid solution in place of catalytic reduction for the removal of the benzylcarbonato group. Benzyl iodide was formed instead of toluene. The optical rotatory power in water was $[\alpha]_{5461} = -21.0^\circ$ for the thiol form and $[\alpha]_{5461} = -107^\circ$ for the oxidised form.

The carbobenzoxy method has also been utilised by Bergmann *et al.* (55) for the synthesis of the peptides, glycyl-, and *l*-alanyl- α -aminoisobutyric acids, α -aminoisobutyrylglycine, *l*-leucyl-*d*-alanine, *l*-leucyl-*l*-alanine, *d*-alanylglycine, and *l*-alanylglycine.

Sifferd & du Vigneaud (56), in reporting a new synthesis of carnosine, record some observations upon the splitting off of the benzyl group from carbobenzoxy derivatives and from benzylthio ethers.

Reduction by sodium in liquid ammonia was found to remove the carbobenzoxy group easily and in good yield even from substances such as carbobenzoxy cystine. This important observation renders the Bergmann-Zervas method applicable to the synthesis of cystine dipeptides. Loring & du Vigneaud (216) have applied this method to the synthesis of cystinyldiglycine, shown to be identical with material derived from glutathione.

Carnosine, β -alanyl-*l*-histidine, occurs in relatively large amounts in muscle but has been little studied physiologically on account of its inaccessibility. The present (56) synthesis, by means of the carbobenzoxy method, affords a 65 per cent yield, based on the histidine used, which is very considerably better than was formerly possible.

Another development of the carbobenzoxy method, due to Bergmann, Zervas & Ross (57), makes possible the synthesis of lysine peptides in which the ϵ -amino group is free as in natural proteins. The key substance is ϵ -carbobenzoxylysine obtained from dicarbobenzoxylysyl chloride.

Abderhalden & Bahn (58) have taken advantage of the ease with which acid removes groups attached to the amino group of serine to synthesise dipeptides in which serine carries the free amino group. For example, *dl*-leucyl-*dl*-serine was esterified and converted into the anhydride. Treatment with acid then opened the ring to form *dl*-seryl-*dl*-leucine. *dl*-Seryl-glycine, *dl*-seryl-*l*-tyrosine, and *l*-seryl-*l*-tyrosine were similarly prepared. During the preparation of tyrosyl serine by the carbobenzoxychloride method the formation of O-N-di-(dicarbobenzoxy-tyrosyl)-serine was observed. A note is also added upon the separation of aspartic and glutamic acid by taking advantage of the differing stability of their benzoyl derivatives.

Turning to the hydantoin synthesis of amino acids, a useful observation reported by Boyd & Robson (59) is that aqueous or alcoholic solutions of ammonium or sodium sulphide or hydrogen sulphide in pyridine act as very convenient agents for reducing unsaturated hydantoins, reduction being nearly quantitative at 100° in twenty-four to seventy-two hours in a closed system or at 58° in seven to fourteen days. Dilute ammonium sulphide for one hundred and twenty hours at 100° hydrolysed the products to amino acids. Piperidine and diethylamine are also cited by the same authors (60) as catalysts in the condensation of aromatic aldehydes with hydantoins. They are conveniently used in pyridine solution and afford good yields.

The synthesis of tryptophane in 60 per cent yield is now reported

by these authors (217), reduction and hydrolysis here being completed in one operation using ammonium sulphide plus ammonium hydroxide.

The condensation of creatinine with aromatic aldehydes in the synthesis of N-methylamino acids is described by Deulofeu & Mendivelzua (61).

Scudi (62) reports the preparation of a number of β -amino acids, while optically active isoserine has been synthesised by Tsunoo (63) from β -chlorolactic acid using the *d*- component.

From Schmidt's laboratory comes an account [Jukes & Schmidt (64)] of titration experiments demonstrating the combination of certain fatty acids with lysine, arginine, and salmine. Several of the compounds of lysine and arginine with higher fatty acids were prepared and described. Greenstein (65) gives details of the synthesis of certain quadrivalent amino acids and their derivatives designed for physical work, and in a second paper (66) describes the synthesis of anhydrolsylglutamic acid amide as the hydrochloride and of ϵ -guanido- α -aminocapronylglutamic acid.

d-Arginine anhydride and *d*-lysine anhydride have been synthesised by Tazawa (67) for enzyme studies, while Carter (68) has prepared a series of phenyl derivatives of α -amino acids having straight or branched chains for use in metabolic experiments.

Bauer, Strauss & Maschmann (69) draw attention to the fact that tyrosine in the free state is only iodinated in alkaline or neutral solution whereas iodination of proteins occurs in acid solution also. Tyrosine amide takes up iodine in acetic acid solution; so also does histidine. It is suggested that a "basic situation" in the molecule is necessary for the introduction of iodine in an acid medium and this is supposed to be achieved in proteins by the peptide linkage in the same way as the acid amide group effects the purpose in tyrosine amide.

The stereochemical relationships of some of the common amino acids have formed the subject of contributions by Freudenberg & Meister (70), who substantiate their former conclusions with regard to *l*(+)-lactic acid and *l*(+)-alanine by a new series of reactions, and by Barrow & Ferguson (71) who establish the relative configurations of valine and alanine. *l*(+)-Norleucine has been shown by Karrer & Itschner (72) to belong to the *l*-series, similar to other naturally occurring amino acids. The configuration of glucosamine is, however, still left in doubt.

Stereochemical relationships in the series of sulphur-containing amino acids are dealt with by du Vigneaud & Patterson (73).

Modification of the method of Schulze and Bosshard by Vickery, Pucher & Clark (74) for the preparation of glutamine from the root of the common beet affords yields of 80 per cent of the total actually present.

The preparation of *d*-glutamic acid hydrochloride from gluten has been studied by Tseng & Hu (75) who report their own experiences in a critical review.

Pyrrolidone carboxylic acid has been obtained by Jodidi (76) by oxidation of the 92 per cent alcoholic extract of Alaska-pea meal and is probably formed from glutamine present. Histidine and arginine were also detected.

STRUCTURAL CONSIDERATIONS

An analysis of the monoamino acid fraction of soybean-protein preparations is reported by Sasaki (77) while Mashino (78) has published figures for the hexone bases and nitrogen distribution. The high histidine-nitrogen figure in Mashino's analysis is noteworthy.

The commencement of work which may throw new light upon the problem of Bence Jones protein is announced by Calvery & Freyberg (79). In their first paper these workers report analyses of two samples of the protein excreted at different times by the same individual—ash, moisture, phosphorus, sulphur; total, amino, amide, and humin nitrogen; tyrosine, tryptophane, cystine, the bases, aspartic and glutamic acids being determined. Further studies are promised which should show by comparison whether the composition of the protein excreted by any individual remains constant and whether those from different individuals are identical. Immunological studies will also be made.

An interesting study of the reversible dissociation of casein, using the ultracentrifuge and the polarimeter, is due to Carpenter (80). The rotation of an original preparation of the protein with molecular weight 96,000 in phosphate buffer at pH 6.8 was measured over the concentration range 0.15 to 1.5 per cent. In the more concentrated solutions the specific rotatory power, $[\alpha]_{D^{20}}$, was -99° and this rotation remained constant upon dilution until a dissociation or breakdown began to occur, as shown by the ultracentrifuge, into molecules of one-third the size, i.e., of molecular weight 32,000. Upon increasing the concentration the change was completely reversible.

White (81) records analyses of purified thyroglobulin, our knowledge of which has also been enlarged by the work of Heidelberger & Pedersen (82). The isoelectric point was found to be 4.58 for the native and 5.0 for the denatured protein. The molecular weight by the ultracentrifugal method was approximately 700,000, the molecules deviating markedly from spherical. Hog and human thyroglobulins are essentially similar. The pH-stability range is 4.8 to 11.3, incomplete splitting taking place at pH 3 and pH 12 [Heidelberger & Svedberg (83)].

The composition of human skin with special reference to the basic amino acids has been studied by Block (84) and Eckstein (85). The ratio histidine : arginine : lysine was found to be 1 : 6 : 7.

As a result of numerous analyses of wool and other keratinous proteins, Block & Vickery (86) defined a keratin as "a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalis, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine, and arginine that the molecular ratios of these amino acids are respectively approximately as 1 : 4 : 12." Block (84) therefore concludes that his material was not a typical keratin. It was obtained from the sole of the human foot or from exfoliated dermatoses and was prepared by peptic digestion and defatting. Reference is made to an analysis by Wilkerson (87) of epidermal scales not subjected to a preliminary peptic digestion from a case of exfoliative dermatitis. The ratios found were 1 histidine : 6 lysine : 15 arginine, which are very much nearer those required by a true keratin. Block suggests that the process of keratinisation might have gone much more nearly to completion in the case of Wilkerson's material. This is hardly borne out by the cystine determinations, if one regards cystine content as an index of keratinisation, Block's material containing 3.4 and Wilkerson's 2.3 per cent cystine, respectively. From Block's definition of his basic "anlage" concept one would rather have expected to find the characteristic ratio in the most individual tissue, namely that with the higher cystine content.

It would seem, as data upon the basic amino-acid content of proteins, determined by accurate methods, accumulates, that recognition must be given to the view that there exists a central basic nucleus, characteristic for any one biological type of protein, around which the remaining amino acids are united. This is of course, in essential, the view put forward many years ago by Kossel. It fell into disrepute

for a time but more recent work, especially that of Block, has compelled its serious consideration, if not acceptance.

The keratins, already referred to, fall into one group of proteins with histidine : lysine : arginine ratios of 1 : 4 : 12. The hemoglobins, Block (88) has shown, exhibit another characteristic ratio, in this case iron : arginine : histidine : lysine, being given as 1 : 3 : 8 : 9, irrespective of the mammalian species from which the crystalline protein was prepared. The ratio of iron to sulphur fluctuates widely, but as the iron content of any hemoglobin is known to be characteristically constant, the fixed ratios of the bases to this element and to one another can only be interpreted in some such way as Block suggests. Recent work makes it probable that the linkage between hematin and globin is through an acidic group in the latter and it cannot therefore be argued that a constancy of proportion between iron and a base, like histidine, is a fortuitous result occasioned by the fact that each molecule of hematin is linked to the protein by means of histidine. The proportionality must have a deeper significance.

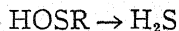
The serum proteins, also, have been shown to exhibit a similar regular relationship. As a result of analyses of the different fractions obtained by salting out serum with ammonium sulphate, and other salts, Block (89) came to the conclusion that the products varied according to the technique employed and were to be considered a result of the disturbance of the normal balance of components present in the serum before the electrolyte was introduced. Such a view is rapidly establishing itself as a result of recent work, although it is difficult to say whether native serum contains only one protein complex or a plurality of interacting members [see Rimington (2) and discussion on "Serum Proteins," p. 140]. Block, Darrow & Cary (90) were able to show that in a series of mammalian sera, although the albumin/globulin ratio, as ordinarily determined, varied within wide limits, the ratios of the basic amino acids present in the total protein material remained constant. Block (91) considers that the proteins of sera should be regarded as members of a general class, analogous to the keratins, and proposes for them the generic term "Orosin." Avian orosin (total protein of the serum) was found to have an arginine : lysine ratio of 10 : 11 as against that of 10 : 18 characteristic of the mammal. Undoubtedly our former conception of an albumin and a globulin fraction is untenable and should be superseded, but it is significant that McFarlane's work with the ultracentrifuge still distinguishes at least three individual "molecular" species

in normal native serum, albeit these do not correspond to the albumin, globulin, etc., ratios found by salting out.

Insight into the structure of the gelatin molecule is afforded by Bergmann's (92) determinations of its glycine, proline, and hydroxyproline content. These amino acids occurred in the ratio 6 : 3 : 2, in good agreement with the assumption that they represent one-third, one-sixth, and one-ninth, respectively, of all the amino acids present, assuming for the latter an average molecular weight of 111. Glycine, proline, and hydroxyproline, it is suggested, occupy some definite periodic arrangement in the gelatin molecule, but the writer cannot help feeling that one is on more dangerous ground here than when dealing with the central basic nucleus idea. Gelatin, of course, is not a well-defined protein to begin with and all the amino acids, were our analyses sufficiently accurate, should show integral proportional relationships.

The form of the sulphur in proteins continues to prove a fruitful field for research. Blumenthal & Clarke (93) suspect some hitherto undiscovered units to be responsible for the fact that several proteins yield sulphate upon treatment with bromine water and sulphide with plumbite while hot fuming nitric acid produces sulphate from another linkage not reacting with plumbite. A word of caution may be ventured since we have evidence to show that the reactions of sulphur groups may be quite considerably modified when their carriers are in peptide linkage with other amino acids [compare Brand & Sandberg (94)].

Freudenberg & Wegmann (95) continue the series of studies upon insulin and in an article dealing with the damage caused by hydrolysis they show that in all probability the sulphur groups are involved in the following series of reactions:



In many instances activity can be restored by the use of suitable oxidising agents, but the changes involved would appear to be somewhat complex. There is a long discussion of insulin chemistry in the light of structural interrelations.

Schock, Jensen & Hellerman (218) recently report that neither cuprous oxide nor phenylmercuric hydroxide, substances easily forming mercaptides, inactivate insulin. Benzoquinone does do so, however, and an interaction with free amino groups is indicated.

That certain metallic elements may enter into the composition of the insulin crystal is demonstrated by Scott & Fisher (96). Precipitation from solutions containing zinc, cadmium, or cobalt ions gave insulin crystals containing a constant amount of the metal. Furthermore, the ash content of each preparation was found to be proportional to the equivalent weight of the metal. A study which may help to throw light upon this phenomenon is that of Main & Schmidt (97) who have investigated the complexes formed between divalent manganese and hydroxy-monocarboxylic acids such as lactic acid, certain dicarboxylic acids, hydroxy di-, and tri-carboxylic acids, the dicarboxylic amino acids such as aspartic acid, sulphuric acid, phosphoric acid, and compounds containing phosphoric acid such as nucleic acid and the glycerophosphoric acids, also certain proteins including casein, edestin, and gelatin. A correlation was found between the number of free carboxyl and phosphoric acid groups and the amount of manganese bound. It would appear that these compounds can all be explained upon the basis of the residual charges residing in such groups.

The importance of sulphydryl groups in the manifestation of proteolytic activity by papain and cathepsin is demonstrated by the work of Purr (98) who shows that loss of activity caused by various agents runs parallel to the disappearance of thiol groupings in the enzyme. Both papain and cathepsin are regarded as proteins with a fixed $-SH$ group, the $-SS-$ form being inactive.

Goddard & Schubert (99) show the essential similarity in action between iodoethyl alcohol and iodoacetic acid on the one hand, and thiol compounds such as cystine, glutathione, wool protein, etc., on the other. The mechanism of inhibition of glycolysis and similar physiological processes becomes ultimately the same, therefore, for these two reagents.

In a paper of general interest, Hand (100) has called attention to the fact that on the basis of viscosity measurements all proteins so far examined can be divided into two groups. Those with viscosities equal to or less than serum albumin can readily be crystallised, while proteins whose viscosities are higher than that of denatured ovalbumin have not yet been obtained in the crystalline state. Hand suggests that this feature may be significant and should be taken into account in developing any theory of protein structure. High viscosity, of course, is usually interpreted as indicating, possibly among other things, a relatively large particle size, and substances of large molecu-

lar or particle weight do not readily form stable crystalline structures.

The question of the presence or absence of diketopiperazine rings in proteins has been actively debated during the past few years and a quantity of empirical data accumulated. In many instances this simply amounted to a demonstration of the *possibility* of producing cyclic structures from proteins by degradation under suitable conditions and with suitable reagents. Ishiyama (101), some time ago, showed that although neutral diketopiperazines are not attacked by either pepsin or trypsin, those with a free carboxyl group could be hydrolysed by trypsin. In a recent communication, Shibata (102) now asserts that diketopiperazine structures with a free amino group attached are similarly hydrolysed by pepsin. Thus glycyl-diaminopropionic anhydride and diaminopropionic anhydride were attacked by the enzyme at the usual optimum pH of 2.4. Certainly these facts will have to be taken into account in the future since one of the strongest arguments against the presence of diketopiperazine rings in proteins was the fact that the neutral diketopiperazines are not attacked by either pepsin or trypsin.

Ssadikow and his coworkers (103, 104, 105) have, by experiment, defined the conditions giving the greatest yield of cyclopeptides from certain proteins with the minimum degree of resinification. For serum albumin, heating in the autoclave with 3 per cent sulphuric acid for five to ten minutes at 220° proved most effective. The whole process of heating and cooling should not exceed three hours.

Some preliminary work has been done upon the fractionation of the products formed and examination of a large bulk of material is promised shortly. It is of interest that Grant & Lewis (106) found no evidence of diketopiperazines amongst the products of the action of 70 per cent sulphuric acid upon silk fibroin for sixty-five to seventy minutes at 30°. Two peptones were, however, isolated, the one with high tyrosine content (12.3 per cent) and low amino nitrogen, the other with higher amino nitrogen and considerably lower tyrosine.

Alcoholysis of casein and gelatin has been carried out by Christomanos (107) using methyl, ethyl, and amyl alcohols at temperatures of 100 to 220°. The degree of splitting was practically the same in each case when the temperature exceeded 180°. A certain proportion of the total nitrogen appeared in the form of volatile compounds, 24 per cent from casein but only 2.2 per cent from gelatin, which rather suggests that it may have had its origin from sulphur-contain-

ing amino acids or from the aromatic structures. The resulting alcoholysate in each case gave a strongly positive reaction for diketopiperazines, said to be present from the initial stages. The biuret reaction was very faint. From casein were isolated leucine anhydride, leucinevaline anhydride, and glycylalanine. Gelatine yielded glycylalanine.

A review of the chemistry of pepsin and trypsin has been contributed by Northrop (108) during the year under review while Lavin & Northrop (109) have attempted to explain, in terms of the absorption of individual aromatic amino acids, the number of narrow bands seen in the ultraviolet absorption spectrum of crystalline pepsin in the region 2500–3000 Å. Gates (110, 111) has also studied the ultraviolet absorption spectrum and established certain very interesting relationships. In the first place it was shown that the total absorption, especially between 2400–2750 Å, increases with the degree of inactivation of the enzyme. The temperature coefficient of inactivation by ultraviolet radiation is very close to unity (1.02) indicating a direct inactivation by the absorbed energy. A similar state of affairs was found by Hussey & Thompson (112) to hold for the inactivation of pepsin by radioactive products. The rate of photo-inactivation is greatest at about pH 2.06, the region in which proteolytic activity is ordinarily manifested. Finally the "destruction spectrum" agrees very closely with the absorption spectrum as was also found to be the case with crystalline urease by Kubowitz & Haas (113, 114) and the wave lengths show marked similarity in the two cases.

Yet another line of attack into the problem of the specific grouping responsible for peptic activity in crystalline pepsin has been directed by Herriott & Northrop (115). Acetylation by ketene was used to try and block the active groups, at pH 4.0 to 5.5, activity decreasing as acetylation proceeded. In all, however, three different crystalline acetyl derivatives were isolated. Ketene is known to react most readily with amino groups of which pepsin has 3 or possibly 4. Blocking of these groups did not appear to interfere with proteolytic action but further introduction of acetyl into situations, from which they were comparatively easily removed by hydrolysis, prevented the manifestation of enzymic activity. These results are in agreement with the less refined experiments of Hugounenq & Loiseleur (116) who used methylating and diazotising agents upon crude pepsin many years ago. Phenolic groups naturally come to mind at once in seeking to interpret the second-stage action of ketene on pepsin and it is of

interest in this connection that the inactivation spectrum already referred to resembles the absorption spectrum of aromatic amino acids like tyrosine (compare also 181).

It may also be recalled that Avery & Goebel (117) found the acetyl group present in Type-I-pneumococcus polysaccharide to be indispensable for the immunological activity of the latter.

The free amino nitrogen of proteins is generally considered to be due only to the ϵ -amino groups of lysine. Zimmermann and his collaborators (118, 119) have devised a method whereby any glycine would be demonstrated, should the amino group occupy a terminal position in a protein or peptide. Methylation should give glycine betaine, distinguishable by its hydrochloride which is sparingly soluble in absolute alcohol. Terminal glycine amino nitrogen was thus demonstrated in a variety of protein split-products and in gelatin after tryptic but not after peptic digestion, but no evidence was found for the presence of such groups in fibrin, egg-albumin, blood protein, or in their digestion products. Benzenesulphonylation followed by hydrolysis has also been used by Gurin & Clarke (120) for the allocation of the free amino groups in proteins, peptides, etc.

The possible occurrence of pyrrole groups in proteins has been the subject of some speculation. Roncato (121) has brought to bear upon this problem a sensitive reagent for pyrrole nuclei, *p*-bromophenylazoxyformamide, the preparation of which is described. By its use small amounts of pyrrolic substances could be detected in tryptic hydrolysates of gliadin, but none in other proteins.

The reagent of Bergmann & Zervas, benzylcarbonylchloride, provides another means of blocking free amino groups in various substances and a means therefore of confirming experiments with phenylisocyanate. Gaunt, Higgins & Wormall (122) have applied this method to insulin and find consequent loss of activity, thus supporting the suggestion that free amino groups are essential for the hypoglycemic effect [compare Jensen (123)]. Reactivation attempts are in progress. Benzylcarbonyl chloride combines readily with native proteins at pH 8 and 5°, the free amino groups disappearing and the protein compound acquiring a new antigenic specificity.

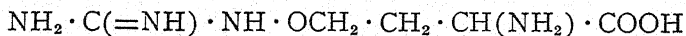
An observation for which no explanation is as yet forthcoming is made by Boyd & Mover (124) to the effect that treatment of edestin with diazotised arsanilic acid results in the entry of arsenic in greater amounts than would be expected on the assumption that only tyrosyl and histidyl groups react. The action of the alkali was excluded as

was also coupling with cyclo-peptides. The first treatment gave approximately normal ratios for arsenic to nitrogen, in agreement with theory, but on subsequent treatment of this material considerably more arsenic was taken up.

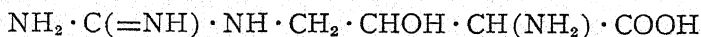
Lieben & Lieber (125) have compared the flavianic acid, arginase-urease, and arginase-xanthhydrol methods for determining arginine and also a colorimetric procedure of Lang based on the reaction with the diketone, acetylbenzoyl. Substantial agreement between all these methods was found and the proportions of arginine split off from proteins in the free state and in large and small peptides has been determined.

In an important paper dealing with the lability of glutamine peptides and the origin of ammonia in tryptic digests of protein, Melville (126) has drawn attention to the fact that the method recently proposed by Chibnall & Westall (127) for the determination of glutamine-amide nitrogen may also include glutaminyl peptides. The peptides *d*-glutaminylglycine, *d*-glutaminyl-*d*-glutamic acid, and *d*-glutaminylglycyl glycine, all having a free amino group in the γ -position with regard to the amide group, were prepared by the Bergmann & Zervas method and shown to exhibit the same instability in aqueous and acid solutions as glutamine itself. The Van Slyke amino-nitrogen values were thus abnormally high. Peptides of the *l*-leucyl-*d*-glutamine type behaved normally.

The amino acid, canavanine, isolated by Kitagawa & Monobe (128) from soybean meal shares with arginine the distinction of being the only known amino acid to yield urea directly in the animal body. The constitution proposed by Kitagawa & Monobe (129) formulated it as the hydroxylamine derivative (I) whereas a possible alternative is formula II, more closely analogous to arginine:



I



II

Canavanine; alternative formulae

Gulland & Morris (130) have sought to distinguish between these structures and have definitely come to the conclusion that canavanine is correctly represented by formula I. An improved method for the

isolation of canavanine is described and its constants are given (m.p. 184° ; $[\alpha]_{D^{20}} = +7.90^{\circ}$). These conclusions are further supported by physical data obtained by Tomiyama (131). The latter determined the dissociation constants of canavanine and canaline (formed together with urea by the action of the tissue enzyme) and found canaline with its substituted $-\text{ONH}_2$ group to be a weaker base than either ornithine or hydroxylamine, while the guanidine group of canavanine is far weaker than that of arginine, but slightly stronger than the imidazole grouping. This is understandable by reason of the modifying action of the oxygen shown on the basic properties of the guanidine nucleus, in the same way as hydroxylamine is a far weaker base than ammonia.

CARBOHYDRATES AND PROTEINS

It is becoming increasingly evident that carbohydrate groups enter into the structure of a wide variety of proteins, not only the so-called glycoproteins (a term of doubtful value) but also into such materials as ovalbumin, casein, the serum proteins, etc. Earlier failure to detect protein carbohydrates was almost certainly due to the use of an unsuitable technique and the modern chapter in this subject may be said to date from the work of Fränkel & Jellinek (132), Levene & Mori (133), and Rimington (134, 135). These workers succeeded in isolating the carbohydrate complex from ovalbumin and the serum proteins, respectively, serum globulin containing as much as 3.7 per cent. It proved to be a glucosaminodimannose, probably polymerised. Sørensen & Haugaard (136) and Sørensen (137) later adapted a colorimetric method to qualitative and quantitative determination (their method gives no colour with glucosamine) and examined a series of protein preparations in each of which they found carbohydrates to be present (Table II).

Since, as pointed out, the colorimetric method employed by these workers does not determine glucosamine, the above figures must be multiplied by 1.4 to represent the total carbohydrate complex, assuming this to consist in each case of a molecule of glucosamine with 2 molecules of a non-nitrogenous sugar. The probability is that separate complexes exist, glucosaminodimannose and glucosaminodigalactose, which may in certain cases be present together in the same protein. Sufficient has been said to make it clear that the original view, emanating from Levene's work, that all mucins and most mucoids are mucoitin sulphuric acid compounds is untenable and that

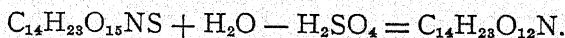
carbohydrate structures may have to be recognised as an integral part of the majority, if not of all, proteins.

TABLE II
CARBOHYDRATE CONTENT OF PROTEINS

Ovalbumin	1.7 per cent; mannose
Easily soluble serum-albumin fraction	0.47 per cent; mannose + galactose
Sparingly soluble serum-albumin fraction	0.02 per cent
Horse-serum globulin	1.82 per cent; mannose + galactose
Casein	0.31 per cent; galactose, no lactose
Lactalbumin	0.44 per cent; galactose, no lactose
Sparingly soluble wheat-gliadin fraction	0.20 per cent; mannose
Egg-white proteins:	
globulin	4.0 per cent; mannose
albumin	1.7 per cent; mannose
conalbumin	2.8 per cent; 3 mannose + 1 galactose
mucin	14.9 per cent; mannose + galactose
mucoïd	9.2 per cent; 3 mannose + 1 galactose

Upon this historical background comes a study by Blix, Oldfeldt & Karlberg (138) of submaxillary mucin, stomach mucin, and cornea mucoïd, all of which contain in the region of 10 to 30 per cent of carbohydrate. Submaxillary mucin contains two complexes—a glucosaminodimannose, identical with that from serum proteins (134, 135), and a greater quantity of a new substance isolated in crystalline form and containing glucosamine—two acetyl groups, a polyoxy acid (not hexuronic) but no sulphur. Its probable formula is given as $C_{14}H_{25}O_{11}N$ and the isoelectric point, pH 2.45. It gives a red colour with Ehrlich's reagent, dimethylaminobenzaldehyde, a reaction which distinguishes it sharply from the known mucoïds. Stomach mucin and cornea mucoïd appeared also to contain two complexes of similar nature.

The writer is struck by a resemblance between the probable composition of Blix, Oldfeldt & Karlberg's material, $C_{14}H_{25}O_{11}N$, and the formula which could be derived from one-half of the chondroitin sulphuric acid molecule [Levene & La Forge (139)] by the elimination of the sulphuric acid group as follows:



The difference is only H_2 in place of O and the fact that the chondroitin fragment contains one acetyl group whereas the German authors report the presence of two in their material. It must be mentioned that Neuberg & Cahill (140) have recently demonstrated the presence in certain bacteria of an enzyme capable of liberating the sulphuric acid completely from chondroitin and mucoitin sulphuric acids and at the same time giving rise to reducing substances.

Using Sørensen & Haugaard's (136) colorimetric method, Grassmann & Schleich (141) have shown that ox-skin collagen contains glucose and galactose in equimolecular amounts and in such a proportion as to give an equivalent weight for this protein of 34,500. Similarly, Schulz & Becker (142) have examined the protein of the ovalbumin glands of *Rana esculenta* and have isolated glucosamine and galactose, present in the ratio 1:1.

A most interesting report is that of Freudenberg & Eichel (143) upon a specific carbohydrate occurring in the urine of individuals belonging to blood group A. This substance was isolated and found to contain, in percentages: carbon, 45.5; hydrogen, 5.9; nitrogen, 5.4; acetyl, 9.5. It yielded glucosamine on acid hydrolysis, the maximum reducing power, reached with *N* hydrochloric acid in three hours, being 50 per cent (as glucose); with snail enzyme, acetylglucosamine was formed; furfural determinations indicated the possible presence of 3 per cent pentosan and 9 per cent uronic acid.

The acetyl group was found to be essential to the activity of the substance, removal of the acetyl by saturated baryta at 100° affording a serologically inactive product which, however, regained its activity on reacetylation.

The writer notes that a calculation based on the nitrogen content indicates a minimum molecular weight of approximately 385, which does not differ greatly from acetylglucosamine + $C_6H_{11}O_5$.

In discussing the isolation of the carbohydrate complex, glucosaminodimannose, from serum protein, Rimington (134) referred to the specific polysaccharides obtained by Heidelberger & Avery from pneumococci, etc., and remarked that

future investigations concerning the immunological rôle of the plasma proteins should not overlook the fact that both albumin and globulin contain carbohydrate material as an integral constituent of their molecules.

We now know that the bacterial polysaccharides act as type-specific haptenes, not antigens, but that for the activity of Type-I-pneumococcus specific polysaccharide, at least, the presence intact of the acetyl

group is essential (117). Some attempts have been made to demonstrate immunological activity with glucosaminodimannose, but so far without success. It may be that, actually, the polysaccharide occurs in the protein in the acetylated form and loses all activity when its acetyl group is removed. This is purely speculation, but since the importance of the acetylglucosamine residue is becoming increasingly evident, trials with an acetylated polysaccharide would seem desirable. Certainly the method of preparation used, splitting of the protein-carbohydrate linkage by long boiling with saturated baryta, would be expected to remove any acetyl groups originally present.

A considerable number of papers has appeared during the year dealing with probable combinations between proteins and colloidal carbohydrate materials such as starch, etc., and the state of carbohydrates in tissues.

Mystkowski (144) considers that the glycogen in muscle is not all in the same state, a part being attached to myosin. Muscles of freshly-killed rabbits were used in the experiments and analyses and fractionations carried out.

Przylecki and his collaborators in a series of papers (145, 146, 147, 148, 149, 150) make out a case for the formation of definite compounds between proteins, dextrin, amylose, etc., and discuss the nature of the groups responsible for these unions. Rafalowska, Krasnodebski & Mystkowski (151) show that the tyrosine-rich silk fibroin and silk peptone both form complexes with amylose, the proportion being about 1 amylose unit to 10 parts of tetrapeptide.

Crystalline dipeptide glucosides have been prepared and investigated by Maurer & Schiedt (152). The difficulty, previously, in the synthesis of such compounds was the removal of the acetyl groups after coupling with amino acid or peptide esters; methyl alcoholic ammonia is now shown to accomplish this. Sarcosylglycine-, *l*-phenylglycine-, and glycylglycine-glucosides were thus prepared. They are stable in aqueous solution but decomposed by acids.

SERUM PROTEINS

The crystallisation of human serum albumin is reported by Adair & Taylor (153), 5 gm. being obtained from 300 cc. of serum, a yield representing about 40 per cent of the albumin present. Recrystallisation was effected by Adair & Robinson's method. Following on this preliminary communication, Wernicke (154) reported that Greenstein, working in his laboratory, had previously obtained crystalline

human serum albumin by following Sørensen's technique. Those of horses, asses, mules, and guinea pigs were also crystallised.

Florkin (155) has determined the plasma-protein content in a number of insects and quotes the following (Table III):

TABLE III
PROTEIN CONTENT OF INSECT SERA

	gm. per 100 cc. serum
Various species of beetles.....	3.18 to 4.12
Lepidoptera larvae	1.3 to 1.8, in- creasing with the age of the larva
Several other species of larvae.....	1.33 to 7.0

The occurrence of a peculiar serum protein in cases of multiple myeloma is described by Jacobson (156). It is precipitated by Hayem's solution and was present in four cases out of seven. The bulk of the globulin in the positive sera proved to be euglobulin and was precipitated as usual by saturation with sodium chloride or magnesium sulphate or half saturation with ammonium sulphate but was not precipitated by carbon dioxide. The material precipitated by Hayem's reagent behaved similarly and was confined to the 14 per cent sodium sulphate fraction. The abnormal euglobulin was distinct from Bence Jones protein, which is not precipitated by Hayem's reagent or by saturation with the above salts.

Paić & Deutsch (157) have investigated the polarimetric determination of serum proteins but find the precision of the method little greater than the familiar refractometric method. Rabbit serum was examined at wave lengths of 5461, 5780, and 6234 and $[\alpha]_D$ established by determinations of rotation before and after precipitation of the protein by heat coagulation. The means found were $-64.7 \pm 2.3^\circ$, $-55.9 \pm 2.0^\circ$, and $-47.5 \pm 1.6^\circ$, respectively. By a second method, where α is obtained by determining the slope of the curve, $\alpha_\lambda = fc$, where c is the concentration in gm. per 100 cc., the values obtained were

$$\alpha_{5461} = -0.638c - 0.12; \alpha_{5780} = -0.516c - 0.33;$$

$$\alpha_{6234} = -0.427c - 0.37.$$

The isoelectric point of crystalline horse serum albumin is found by Sandor (158) to be independent of the concentration but dependent upon the presence of neutral salts. Roche & Marquet (159),

applying Adair's osmotic pressure method to horse-serum albumin at 0° and pH 7.4, find a molecular weight of 69,000 for this protein. A fifteen-year-old sample gave the same value.

Two papers of importance are presented by Bellis & Scott (160) and Lehman & Scott (161). These workers find that there is a shift of protein from cells to plasma under certain conditions; addition of isosmotic dextrose, sodium chloride, calcium chloride, or barium chloride to oxalated or defibrinated blood *in vitro* caused an increase in the plasma-protein concentration proportional to the degree of dilution. Hyperosmotic solutions caused a decrease while the non-protein nitrogen was found to increase in each case. It was chiefly the albumin which was affected and pH determinations showed that the phenomenon could not be accounted for on the basis of hydron change. The surface tension of the blood was not appreciably altered.

Bellis & Scott summarise their conclusions as follows:

From the results of our experiments, we believe that the lowering of the erythrocyte and plasma protein potentials in the presence of hyperosmotic solutions results in adsorption of the proteins on the erythrocytes It seems that the contents of the red blood cells are probably in concentration equilibria with the contents of the plasma, both colloid and crystalloid, necessitating changes in the contents of the corpuscles corresponding in equivalence to alterations of the fluid content of the blood.

In illustration of this equilibrium, Lehman & Scott (161) show that the protein contents of the plasma and serum derived from the same blood are practically identical notwithstanding the 0.2 to 0.3 per cent of fibrinogen present in the former. The extra protein in the serum was chiefly albumin.

The dependence of the solubility of the plasma proteins upon such factors as pH, temperature, and lipid content when in concentrated solution of potassium phosphate and the bearing of these results upon the separate precipitation of the plasma proteins is discussed by Butler, Blatt & Southgate (162).

Lustig & Mandler (163) continue the studies upon the serum-protein subfractions emanating from Lustig's laboratory in Vienna. A résumé of the recent results and of the methods underlying the fractionation are to be found in (2). In the present instance, the tyrosine and cystine contents of the proteins from puncture fluid in cases of hepatic cirrhosis, sarcoma, and carcinoma have been studied.

With the increasing use of the ultracentrifuge for the determination of molecular weights of proteins and the analysis of mixtures,

it is undoubtedly fortunate that the theory underlying the principles involved should receive thorough treatment. Lansing & Kraemer (164) have provided such a study [see also (7)]. They distinguish between number-average, weight-average, and what they term Z-average molecular weights as calculated from sedimentation-equilibrium data.

Using the ultracentrifuge and a modification of Lamm's refractive index method for working with solutions of proteins in high concentration, McFarlane has conducted a series of investigations of the greatest importance upon serum proteins and native serum. Recrystallised horse serum albumin was found (165) to be monodisperse over a wide range of concentration whereas the reprecipitated globulin fraction was always associated with both larger and smaller molecules. When native serum (horse) was examined, it was found that there was present about 80 per cent of a light fraction together with 20 per cent of a heavier fraction although the albumin/globulin ratio, as ordinarily determined, was unity. On dilution of the serum, a redistribution took place, the proportion of lighter to heavier molecules eventually becoming 1. Conversely, starting with mixtures of "albumin" and "globulin" and increasing the concentration, the proportion of the light to the heavy fraction increased until a state of affairs was reached similar to that in native serum.

There is thus a marked interaction between the component proteins of serum. The lighter fraction can, apparently, be regarded as identical with "serum albumin" as ordinarily isolated. The dilution effect is most marked with horse serum but the same phenomenon was found (166) to occur in human and bovine sera. Three protein types are distinguishable in every case, the third present in small concentration only, but the differences are purely quantitative between the different sera.

In a complementary investigation upon urinary proteins, McFarlane (167) found that, in three out of five cases examined, only albumin was present; in the remaining two, a mixture of albumin and globulin. In every case, however, the high power of boundary spreading evinced by the albumin suggested that it was polydisperse and the same was found to be true of the blood-serum albumin of the individuals in question. It is interesting that only those (two) individuals showing marked signs of renal disease passed globulin as well as albumin into the urine. Applying these findings to the examination of a number of pathological sera, McFarlane (168) has pointed out

the constancy in the relative proportions of the three protein types present in the serum of healthy persons, irrespective of the total protein concentration, and deviations from this ratio as a result of disease.

After a careful perusal of the paper, it still seems to the writer, however, that only the fringe of the problem is being touched. As McFarlane himself carefully points out, there appear to be substances present in certain pathological sera, and to a lesser extent, even, in some normal sera, which retard the sedimentation of the protein particles. It is thus impossible to tell whether or not the proteins present have abnormally low molecular weights, although the fact that they are often equally affected, their *relative* sedimentation constants remaining undisturbed, supports the view that they are of the normal molecular dimensions but sedimenting abnormally. The occurrence of this phenomenon is a serious handicap to interpretation of the results obtained; nevertheless McFarlane has made it abundantly clear that the state of affairs ruling in normal serum is very different from what would be inferred from determination of the albumin/globulin ratio in the usual way.

It would appear from the present work that two main proteins of the serum must be recognised, the A and G fractions, as McFarlane calls them to distinguish them from the albumin and globulin obtained by salting out, and it would be an interesting development to determine the ratios of the bases in the simple A protein obtainable, unmixed with G, from those urines shown ultracentrifugally to contain only the A fraction (see above). One could then deduce the composition of G by calculation.

Whether this method of serum investigation will ultimately prove to be of value in the diagnosis and elucidation of disease, it is at present difficult to say. Certain difficulties still remain to be overcome before unequivocal interpretations can be given to the experimental results. The method, at present, is feeling its way but the possibilities for the future would appear to be promising.

It is of interest that in a case of myeloma, a fourth protein fraction in the serum was observed, and, as mentioned previously in this section, Jacobson (156) has also reported the occurrence of an abnormal protein detectable by salting out and precipitation reactions in several cases of the same disease.

In a final paper, McFarlane (169) has studied the behaviour in the ultracentrifuge of serum fractions obtained by salting out. The outstanding feature of the results was the lack of uniformity of the

globulin fraction. While in some cases, e.g., normal horse- and some pathological human sera, the proportion of albumin coming down with the globulin was small; in others, such as normal human- and cow sera, the globulin fraction obtained by half saturation was grossly admixed with albumin.

It is somewhat difficult to effect a comparison of these new findings with Sørensen's theory of reversibly dissociable systems. Had this theory held for the conditions appertaining in normal serum, one would have expected a dissociation, on dilution, of heavier into lighter particles whereas in actual fact the reverse occurs. Again, if the units of the protein were a number of loosely bound polypeptide chains, mutually attracted by residual affinities, one would not expect to encounter in all sera two or three main types, having very different particle sizes, and present in constant proportions (where no dilution has occurred).

No doubt we are still ignorant of the peculiar conditions operating in serum and causing its constituents to deviate from the laws applicable to simple solutions. Only further research will serve to define these conditions.

PHYSICAL CHEMISTRY

A thorough investigation has been carried out by Linderstrøm-Lang (170, 171) of preparations of clupein obtained from herring sperm. Being a comparatively simple protein, it was to be expected that the titration data would throw light upon its structure. In all, six fractions were studied and these found to be very similar to one another and to an unfractionated preparation, the curves for all closely resembling that of a simple aliphatic monoamino acid or simple peptide. The values for P_b and P_s (the logarithms of K_b and K_s) varied between 2.9 to 3.3 and 7.4 to 8.0, respectively. This protein would appear to have a mean molecular weight of 4,000 to 4,100, thus containing 19 to 20 arginine molecules and 7 to 8 monoamino acids, to possess 1 free amino and 1 free carboxyl group per molecule, and to be representable as an unbranched polypeptide chain.

The viscosity of gluten solutions in various solvents and the effect of various influences upon this property has been the subject of a good deal of study by Cook & Rose and their collaborators (172, 173, 174, 175). Many of the earlier observations are explicable in the light of the finding (175) that gluten very readily suffers hydrolysis in dilute alkaline or acetic acid solution, even at 0°, although urea

and sodium salicylate have no apparent effect, even on heating. Something analogous to the "coagulation point" of albumins and globulins was observed when gluten, in either of the latter solvents, was heated progressively. In urea solution, the viscosity decreased up to 70° then increased, to be followed again by a decrease, while the sodium salicylate solutions increased in viscosity throughout the whole period of heat treatment up to 80°, when a decrease occurred. Turning to the experiments at ordinary temperatures, it was found (172) that in urea solution the viscosity was constant over a range of pH 6.1 to 9.2, outside of which instability occurred.

The glutens obtained from different flours had essentially the same viscosity in acid or alkali but markedly different viscosities in urea or sodium salicylate, these differences being partially correlated with quality. An ageing phenomenon was, however, encountered (172), even in the organic solutions, being more marked in acid or alkali. The reason for this falling-off in viscosity would seem to be obscure. It cannot be occasioned by hydrolysis since no such action was found (in the organic solvents) even at elevated temperatures (175). One can only conclude that some sort of disaggregation occurs and in this connection the paper by Blagoveschenski & Yurgenson (176) is of particular interest. They conclude, as a result of their experiments upon wheat proteins and wheat flour, that there are present in the latter enzymes which cause a disaggregation of wheat proteins, a change which is quite distinct from hydrolysis since it is unaccompanied by any increase in free amino nitrogen. The optimum reaction would appear to be at pH 8.5. Should the presence of such enzymes be confirmed an explanation might be forthcoming of the gradual decrease in the viscosity of gluten solutions on standing and the degree of this change might be correlated with the quality of the flour in question.

Van Manen & Rimington (177), studying the phenomenon of watery-whiteness in eggs, could find no evidence for the presence of proteases (although ereptases were present in the thick white) and suggested, as one possible explanation of the phenomenon, that a similar disaggregating enzyme might be present in those eggs which suffered the watery-white transformation. The recent publication of Samuel (219) should, however, be referred to in connection with this topic. He did not confirm the findings reported (176) but the conditions prevailing in his experiments were widely different.

Fractionation of gluten by salt solutions has been carried out by

McCalla & Rose (173) who find about 10 to 15 per cent of a very soluble fraction associated with a remaining complex which can be divided into subfractions of differing solubilities and chemical composition. When redispersed and combined, the constituent fractions re-form a typical gluten.

The application of the surface-film method of Adam & Langmuir to the spreading of proteins has been developed during the past few years by Gorter and it would appear that these painstaking researches are now beginning to bear a rich harvest. Proteins spread like other substances whose solubility in water is neither too great nor too small and in each case the maximum area covered at the isoelectric point (where the protein behaves as an un-ionized body or zwitterion) approximates to 1 sq. m. per mg. It is significant that the same value holds for proteins belonging to classes whose molecular weights are multiples of 34,500; in other words, the forces bringing about surface orientation and spreading are sufficient to overcome the intermicellar cohesion responsible for the aggregation of the fundamental 34,500 particles (Svedberg's unit molecular weight) into molecules of 2, 3, or 6 times this size [compare Rimington (178)].

A description of an improved form of apparatus is now given by Gorter & Seeder (179), while in a study of the spreading of complex proteins, Gorter, van Ormondt & Meijer (180) compare the spreading at either side of the isoelectric point of ovalbumin and the complex obtained by combining this protein with tartrazine (a strongly acidic substance). It was found that little effect was noticeable on the alkaline side but that the minimum around pH 2.7 to 3.0 was completely abolished. The explanation advanced is that the amino groups of the protein, now combined with tartrazine, are unable to function ionically; the protein has become an acidic substance. The minimum quantity of tartrazine necessary to produce this effect can be determined and corresponds to 39 free amino groups per ovalbumin molecule. Similarly, by adding spermidine (strongly basic) to pepsin, suppression of the alkaline minimum is achieved. That the interpretation of these effects is correct is substantiated by the observation that nucleic acid, itself an ampholyte, in no way influences the shape or character of the pH-spreading curves. Neither does coupling with an azo compound produce any detectable difference.

Pepsin and trypsin are saturated by small quantities of tartrazine and spermidine, respectively, tartrazine indicating 5 amino groups per molecule in pepsin, in good agreement with analytical finding; the

above-named proteins would appear to be themselves complexes in which a multivalent acid has combined with most of the amino groups in the case of pepsin, or a multivalent base with a number of acid groups in the case of trypsin. The effect of ions upon the spreading of pepsin and trypsin has been further reported by Gorter (181).

Myosin does not ordinarily spread readily on a buffered water surface, it being too little soluble. If, however, very small quantities of trypsin are added to the liquid, spreading is facilitated and it becomes possible to measure the areas of the surface films before hydrolysis causes them to decrease. The conditions governing this phenomenon have been studied by Gorter & van Ormondt (182) and it is shown that a definite time is taken for the trypsin to exert its full effect. The dependence of this time on temperature affords a temperature coefficient of 3.6 for 10° , and the relation between temperature and the logarithm of trypsin concentration is shown to be linear.

These results are of particular interest in demonstrating that an enzyme can, by inducing a protein to increase its surface, prepare it for hydrolysis into smaller peptides and amino acids. Most probably, a union of protein with enzyme takes place. In an analogous manner the extended spreading of ovalbumin on the acid side of its isoelectric point could be achieved by blowing it onto the surface of the bath in which tartrazine was already dissolved.

Schulman & Hughes (183), further investigating an apparent difference in action between crude pancreatin preparations and purified enzymes upon protein films, find that there is present in the crude material a protein-fatty-acid complex which behaves in a manner similar to pancreatin itself but is not destroyed when the latter is inactivated. These interfering capillary-active substances have also the power of penetrating and modifying surface films. Such displacement and complex formation has been studied in further detail (184) in the case of long-chain alcohols, acids, glycerides, sphingosine, and psychosine.

Duce (185) observed no change in the refractive index of certain proteins on heating nearly to the coagulation point; above this point, however, an increase occurred.

Djatschkowsky & Liwanskaja (186) have studied the gel formation occurring when ovalbumin is shaken repeatedly with ether. The maximum formation occurs with 0.5 per cent of ether and the quantity of solid matter in the gel is inversely proportional to the albumin concentration. The material so extracted gives a positive biuret, but

no xanthoproteic reaction while the residual sol gives both. It is concluded that ether removes the polar constituents. The formation of protein gels with acids has also been studied by Kopaczewski (187) while the swelling of structural proteins, collagen, etc., in water and dilute acid has been investigated by Jordan-Lloyd & Marriott (188).

The electrical forces operating in the adsorption of gliadin at a glass-liquid interface have received treatment from Martin (189). It was found that the preparations used contained both positively and negatively charged particles. Adsorption is a process of electroprecipitation, the free amino and carboxyl groups remaining directed into the liquid, in which position they are still capable of reaction. The conception of proteins as colloidal electrolytes has been discussed by Jordan-Lloyd (190).

The effect of irradiation with α -particles upon egg albumin is to produce coagulation only if the initial pH is at the isoelectric point [Arnow (191)]. Oxygen is produced by the decomposition of water and is utilised by the protein. Reaction with hydrogen also occurs to a slight extent and in at least two distinct mechanisms. Irradiation of solutions of pH greater than the isoelectric point causes a fall in pH, otherwise there is little effect. The ultraviolet absorption is increased at and below the isoelectric point but decreased if above, and the effect upon viscosity is similar. The influence of ultraviolet radiation upon proteins and peptones has been studied by Lieben & Jesserer (192).

The observation of Rondoni & Pozzi (193) that the addition of hydrogen peroxide to a system containing protein, such as an autolysate or serum, causes an increase in the amount of material precipitable by trichloroacetic acid, has been confirmed and further investigated by these workers (194). It is considered to be a colloid-chemical phenomenon, an alteration in the state of aggregation being brought about, and this suggestion is borne out by the finding that the limits of the precipitin reaction are not altered. In view of the discussions in this review on the proteins of native serum, the effect of ions in protein systems, etc., such an explanation would appear to be quite feasible.

The degree of polymerisation of a variety of proteins in solution has been measured by Piettre (195), using the adsorption of the protein by activated charcoal in a quantitative manner.

Smith (196) records the effect of variations in ionic strength upon the apparent isoelectric point of egg albumin, determined by

cataphoresis. An inverse relationship between isoelectric point, ionic strength of the buffer, and protein concentration was established and by extrapolation the value for 0.1 per cent ovalbumin at zero ionic strength was found to be $\text{pH } 4.85 \pm 0.01$ at 25° . Plotting similar values for other concentrations against protein concentration affords a straight line which at "zero protein concentration" indicates an isoelectric point of 4.86 ± 0.01 .

The peptisation of edestin by salt solution does not, according to Holwerda (197), reach its minimum at the isoelectric point and this method is therefore inapplicable for the determination of isoelectric points. Peptisation is a colloid-chemical phenomenon and the action of anions upon viscosity is found to follow the same order. Wells, Miller & Drake (226) describe a rapid and reliable method for the determination of the osmotic pressure of protein solutions.

By extrapolation of the equation, $n_p - n_s = aC$, to solutions containing only protein, Hand (199) calculates values for n_p (refractivity of the protein in the dissociated state) thus permitting the comparison of one protein with another. The specific refractive increment, a , is not characteristic of the protein but varies with the refractivity of the solvent. Values of n_p are given for a large number of proteins.

From Cohn's laboratory have appeared further studies (200) of multivalent amino acids and peptides, the dielectric constants and electrostriction of the solvent being reported for solutions of tetrapoles. In the case of diaminodithiodicaproic acid, the two dipoles are of the same length and a twisting of the chains occurred with consequently relatively small effect on the dielectric constants of solutions or on electrostriction of solvent. Lysylglutamic acid was selected as comprising a small dipole and a longer one, the latter between the ϵ -amino group of lysine and the γ -carboxyl group of the glutamic acid residue. Here the effects upon dielectric constant and electrostriction of solvent were maximal. McMeekin, Cohn & Weare (201) have prepared derivatives of amino acids which no longer possess zwitterion structure and find the apparent molal volumes higher than those of the parent amino acids. Whereas the solubility of amino acids in alcohol-water mixtures varied in the same manner as salts, the solubility of these amino acid derivatives corresponded to that of other uncharged organic molecules.

The distribution coefficients of many α -amino acids between butyl alcohol and water at 25° have been measured by England & Cohn

(202) and the factors affecting solubility studied in relation to structure.

Surprisingly little accurate thermal data exist in connection with amino acids and similar biologically important substances. This gap has been partly filled by Edsall's (203) determinations of the apparent molal heat capacities of a series of amino acids and organic compounds in aqueous solution and in the solid state. Polar substances like glycerol, urea, dextrose, etc., obeyed the law of ideal solution, having the same molal heat capacity in water as in the crystalline state. In the case of non-polar substances, it was found that the CH_2 group caused a larger increment in apparent molal heat capacity in solution than in the pure form. The heats of combustion of *l*-cystine, *l*-cysteine, β -thiolactic acid, and $\beta\beta'$ -dithiolactic acid have been determined by Huffman & Ellis (204), while Zittle & Schmidt (205) report the heats of solution and of dilution, and the specific heats of aqueous solutions of certain amino acids.

The solubilities and differential heats of solution of *d*-tyrosine, *dl*-tyrosine, di-iodo-*dl*-tyrosine, dibromo-*l*-tyrosine (hydrated and anhydrous), and dichloro-*l*-tyrosine (hydrated) have been determined by Winnek & Schmidt (206). Evidence is brought forward indicating the compound nature of *dl*-tyrosine. The apparent acid and basic dissociation constants for dibromo-*l*-tyrosine and the dichloro derivative have been determined at 25° and 40° from solubility determinations and the apparent heats of ionisation have been calculated. The allocation of the dissociation constant to each particular group is discussed.

A comparison of the expected conductivities of aqueous solutions of glycine, *dl*-valine, and *l*-asparagine, based on thermal data by Mehl & Schmidt (207), with the values actually found shows good agreement.

Alanine is shown to have no effect upon the ionic atmosphere of potassium chloride solutions, the charges of the zwitterion molecule being sufficiently near to result in behaviour like that of a neutral molecule. The activity coefficients of glycine and valine in presence of zinc, sodium, and thallium chlorides have recently been determined by Joseph (220, 221).

Dalton & Schmidt (208) have recorded the solubilities in water at 0 to 75° and at 100° of *l*-asparagine hydrate, *l*-cystine, *d*-isoleucine, *dl*-methionine, *l*-phenylalanine, *dl*-serine, taurine, and *l*-tryptophane and the densities of their aqueous solutions at 25°. The heats of

solution have been calculated from this data. The active forms of isoleucine and phenylalanine are more soluble than the inactive forms, probably indicating the racemic nature of the latter.

In a study of the reaction between formaldehyde and simple amino acids, Tomiyama (209) shows from equilibrium-constant data that between pH 10 and 8 the reactants combine in molecular ratios. The mechanism of the reaction is discussed and it is suggested that the reaction product is possibly a molecular rather than a methylene compound.

The equilibria of the basic amino acids in the formol titration has been especially studied by Levy (210) who finds that their behaviour may be interpreted on the same basis as that of the simple amino acids. The imidazole group reacts only slightly; the reaction with arginine is very slow.

The titration constants of a number of acid amides and dipeptides have been determined at 25° by Melville & Richardson (211) and it is pointed out that in certain peptides the constants attributable to the amino group are unusually low, a fact which might lead to erroneous results from formol or alcohol titrations. The equilibria which exist between amino acids and certain aromatic aldehydes have been studied very thoroughly by Gulland & Mead (212).

The hydrolytic decomposition of phenylalanine in water at 80° to 110° has been confirmed and the equilibrium of the system studied by Baur & Schindler (222).

Dem'yanov & Putokhin (223) show that the action of nitrous acid on tryptophane is to produce nitroso- β -indoleacrylic acid.

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ONDERSTEEPOORT VETERINARY RESEARCH LABORATORY
PRETORIA, SOUTH AFRICA

THE CHEMISTRY AND METABOLISM OF COMPOUNDS OF SULFUR*

BY VINCENT DU VIGNEAUD AND HELEN M. DYER

*From the Department of Biochemistry, School of Medicine,
George Washington University, Washington, D.C.*

Newly isolated sulfur compounds.—Since this field was last reviewed, the structures have been announced for two additional sulfur-containing compounds, asterubin and djenkolic acid, isolated from natural sources. Asterubin, which was isolated by Ackermann (1) from starfish, was found to yield guanidine upon oxidation, and after hydrolysis dimethylamine and carbaminytaurine. From this and other properties the structure $\text{HN}:\text{C}(\text{NHCH}_2\text{CH}_2\text{SO}_3\text{H})\cdot\text{N}(\text{CH}_3)_2$ was assigned to it. This was confirmed by synthesis through the condensation of taurine with dimethylcyanamide (2). A much improved synthesis was later presented in which cystamine was condensed with dimethylcyanamide and the tetramethyldiguanylcystamine oxidized to asterubin (3). The compound was found to cause a marked increase in blood sugar but it had no effect upon blood pressure (4).

The very interesting compound, djenkolic acid, which is related to cysteine, was isolated by Van Veen & Hyman (5, 6) from the djenkol bean. From the ultimate analysis of the compound and its various derivatives, and from the fact that cystine was obtained by the action of concentrated sulfuric acid, they concluded that djenkolic acid possessed the structure $\text{CH}_2: [\text{SCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}]_2$, which may be regarded as the cysteine-thioacetal of formaldehyde. The isolation of djenkolic acid is of particular interest because it may possibly be responsible for a portion of the non-cystine non-methionine sulfur in certain proteins, particularly those of other leguminous plants.

Synthetic studies.—The outstanding synthetic contribution of the period under review is unquestionably the synthesis of glutathione by Harington & Mead (7) which confirmed the structure generally accepted for this tripeptide, namely γ -glutamylcysteinylglycine. The ingenious carbobenzoxy method of Bergmann & Zervas (8) was utilized in achieving this synthesis, but in place of using the catalytic reduction with hydrogen and palladium to remove the carbobenzoxy group, they resorted to reduction with phosphonium iodide. As stated

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by the authors, the yield was not encouraging from a preparative standpoint, but sufficed for structural proof. The cysteinylglycine ethyl ester hydriodide used in the preparation was made by the condensation of dicarbobenzoxy cystinyl chloride with glycine ethyl ester, followed by reduction of the condensation product with phosphonium iodide. The α -methyl carbobenzoxy glutamyl chloride was then condensed in chloroform in the presence of diethylamine with cysteinylglycine ethyl ester hydriodide. Saponification of the ester and reduction of the carbobenzoxy group with phosphonium iodide resulted in the formation of glutathione, which was isolated as the copper salt and regenerated in the usual manner.

Cystinyldiglycine has now been obtained in crystalline form, the compound having been prepared from dicarbobenzoxy cystinyldiglycine by reduction of the latter in liquid ammonia with metallic sodium followed by oxidation of the cysteinylglycine so formed (9). The S-benzyl derivative of cysteinylglycine was also obtained in crystalline form by benzylation of the latter in liquid ammonia. The synthetic cystinyldiglycine and benzylcysteinylglycine were found to be identical with the corresponding compounds obtained from the cysteinylglycine prepared by the partial hydrolysis of glutathione by the method of Kendall, Mason & McKenzie (10), thus affording synthetic proof of the identity of this dipeptide isolated by them from glutathione. It has also been shown that the benzyl group can be removed from S-benzylcysteine by reduction in liquid ammonia with metallic sodium, thus introducing a means of covering, for various purposes, the SH group with a radical that can be readily removed (11).

The structure originally proposed for homocystine (12) has now been confirmed by synthesis (13). A malonic ester amino acid synthesis employing benzyl- β -chloroethyl sulfide was used and the benzylhomocysteine so obtained was converted by reduction and subsequent oxidation to homocystine. The synthesis, in addition to proving the structure, serves as a method of preparation more convenient than that hitherto available. Previously it has been necessary to prepare methionine and then convert it to homocystine by the action of strong sulfuric acid. The free homocysteine has also been isolated in crystalline form and its conversion to the thiolactone has been studied (14). The isolation of the hydriodide of the thiolactone of homocysteine formed by the action of hydriodic acid on methionine by Baernstein (15) has not only been confirmed but the compound has been prepared directly from homocysteine by the action of hydriodic acid (14).

The preparation of both optical isomers of homocystine has now been accomplished and their spatial relationship to the optical isomers of methionine demonstrated (16). *L*-Homocystine with a specific rotation of $+77^\circ$ in hydrochloric acid was converted by methylation into the naturally-occurring methionine. The resolution was accomplished by means of the brucine salt of the *N*-formyl-*S*-benzylhomocysteine and the *d*- and *l*-benzylhomocysteines from these brucine salts were converted to the corresponding homocystines.

The sulfur-containing moiety obtainable from vitamin B₁ by the Williams sulfite cleavage (17) has been shown to be 4-methyl-5- β -hydroxyethylthiazole by the work of Clarke & Gurin (18). It was synthesized by condensing methyl- α -chloro- γ -ethoxypropylketone with thioformamide, splitting the resulting ether with hydrochloric acid to give 4-methyl-5- β -chloro-ethylthiazole and then hydrolyzing the latter with water. It was also shown that the acid, C₆H₅O₂NS, obtained by Windaus and collaborators by the action of nitric acid upon vitamin B₁ is the 4-methylthiazole-5-carboxylic acid. From the stability of the sulfur of the vitamin and of the thiazole cleavage product and its quaternary ammonium salts, evidence was obtained which supports the view (17) that the thiazole exists in the vitamin in the form of a quaternary salt.

The preparation of cystinehydantoin in excellent yield has been reported by Hess (19). The compound gave a negative Sullivan reaction but, as would be expected, positive Folin-Marenzi and Okuda reactions.

In a study of multivalent amino acids, Greenstein (20) synthesized ϵ, ϵ' -diamino-di-(α -thio-*n*-caproic acid), an isomer of hexocystine. In an endeavor to extend the studies on guanidocystine, Greenstein (21) has also prepared the corresponding cyamidene derivative of cystine, namely anhydro- α, α' -diguanido-di-(β -thiopropionic acid). As would be expected from the behavior of analogous substances it was found that the cyamidene derivative is extremely labile to even low concentrations of alkali, decomposing very rapidly to yield alkali sulfide, pyruvic acid, and guanidine.

A renewed interest in the general organic chemistry of sulfur compounds has been quite apparent during the past year, and many syntheses of other sulfur-containing organic compounds have been reported. A few of the syntheses closest to biochemical interests will be mentioned. The preparation of the *cis* and *trans* β -sulfoacrylic acids (22), of β, β -disulfopropionic acid (23), and of β, β -disulfobu-

tyric acid (24) has been described by Backer & Beute, and β,β -disulfoglutaric acid has been prepared by van der Zanden (25). Garforth & Pyman (26) have described the preparation of 4(5)- β -alkylaminoethyl glyoxalines and certain of their derivatives. The preparation of aminohydroxypropane-sulfonic acid and its derivatives has been reported by Tsunoo (27) and the synthesis of thiazole compounds has been described by Horii (28).

In-vitro oxidation-reduction studies.—The question of the oxidation and reduction of disulfides has continued to attract the attention of a considerable number of workers. According to Shinohara & Kilpatrick (29) the oxidation of cystine to cysteic acid by iodine in acid solution is practically quantitative and the rate-determining step is the reaction between a molecule of cystine and a molecule of iodine, which remains almost constant over a range of pH 1 to 5, but is affected by an increase in the concentration of iodide within a limited range. It appears probable that the triiodide ion is the reactant. In a continuation of his studies on the question of the path of oxidation of cystine, Toennies (30) has indicated that the chief reaction product of the oxidation of cystine by permonosulfuric acid is a monosulfoxide when the ratio of the reagent to the cystine is 1.01 to 1, and when this ratio is 2.10 to 1, a disulfoxide is obtained. Lavine & Toennies (31) have isolated a disulfoxide from the oxidation products resulting from the oxidation of cystine perchlorate in acetonitrile with perbenzoic acid. The oxidation of dithio acids to sulfonic acids by various oxidizing agents is being studied by Preisler & Preisler (32, 33) in the hope of solving the problem of the mechanism of this complex oxidation. Their experimental data indicate that the rate-controlling step in the reaction between thallic sulfate and cystine in sulfuric acid solution is a second-order reaction in which the rate is approximately proportional to the concentrations of cystine and thallic sulfate and is affected by an increase in the sulfuric acid concentration. The rapid oxidation of cystine to cysteic acid by bromine and the relatively slow oxidation of cystine to cysteic acid by iodine were also considered. Thimann (34) has presented an interesting discussion of the mechanism of the oxidation of cysteine and cystine, and the rôle of oxidation-reduction in cell metabolism. In a subsequent paper (35) he has also discussed this question in connection with the findings of Fruton & Clarke (36) and with the observations of Huffman & Ellis who have recently presented thermal data on the heats of combustion of the sulfhydryl and disulfide forms of cystine and

β -thiolactic acid (37). The heat capacities, entropies, and free energies of these same compounds have also been determined (38).

In a continuation of their studies of the characteristic disulfide properties of keratins, Goddard & Michaelis (39) reduced the keratin of wool by sodium thioglycolate to the sulfhydryl protein (kerateine). The hydrogen of the $-SH$ group of kerateine was substituted by treatment with iodoacetate and similar compounds. The kerateine was also oxidized to metakeratin at neutral reaction by air or by ferricyanide. None of the derived keratins was obtained in a strictly pure state, nor were any of them considered as chemical entities. All of the derived keratins, unlike the parent keratin, were readily digested by trypsin and pepsin, suggesting that once the disulfide bond is broken the protein becomes digestible by true proteases no matter what other reactions the sulfur may undergo. No loss of sulfur occurred under proper preparation of the derivatives, but they all differed distinctly in their solubilities and in their isoelectric points from the original protein.

Additional chemical studies.—In an effort to throw light on the possibility of the assumption that H_2S or CH_3SH may add to methylene pyruvic acid as an early stage in a probable biological synthesis of methionine and homocystine, Nicolet (40) has demonstrated that the addition of benzyl- and *p*-tolylmercaptans to $\alpha:\beta$ unsaturated ketones takes place very readily at 100° without catalysts, while similar additions to $\alpha:\beta$ unsaturated esters occur rather less readily and in the presence of piperidine. Toennies & Elliott (41) were unable to verify previous statements regarding the constitution of cystine phosphotungstates but found that the crystalline precipitates varied in their composition according to the component ratios used. The optimal conditions for the precipitation of *L*-, *dl*-, and *m*-cystine by phospho-12-tungstic acid were ascertained.

Diphenyldithiodiglycolic acid has been shown to be hydrolyzed by alkali to yield 50 per cent of $PhCH(SH)CO_2H$ and 45.5 per cent of $PhCOCO_2H$, the latter having been formed with the loss of hydrogen sulfide from the unstable intermediate $PhCH(SOH)CO_2H$. This work was undertaken by Schöberl, Berniger & Harren (42) in an effort to explain the mechanism of the splitting off of hydrogen sulfide from cystine by alkalies, which has been the subject of many interesting studies in the past few years. Oxygen was consumed chiefly in oxidizing the sodium sulfide to $Na_2S_2O_3$. Schöberl & Eck (43) have also noted that when they heated dithiodiglycolic acid

and dithiodisuccinic acid in an acetate buffer solution or their sodium salts in water, hydrogen sulfide and sulfhydryl compounds were formed.

In an extensive study of the solubilities of certain amino acids and related compounds in water Dalton & Schmidt (44) have determined the solubilities of cystine and methionine, the densities of their solutions at 25°, their calculated heats of solution, and partial molal volumes.

The apparent identification of heparin as a chondroitintrisulfuric acid by Jorpes (45) will not be discussed in detail since Schmitz (46) at almost the same time claimed to have obtained a highly purified preparation of heparin which contained no sulfuric acid. It had about one-tenth the amount of ash of the preparation reported by Jorpes and did not form the hydrochloride salts. Only further work will disclose whether heparin is a sulfur compound.

Methods of determination.—Additional modifications of the current titration and colorimetric methods for the determination of sulfhydryl and disulfide compounds continue to appear. The changes suggested have usually resulted from efforts to avoid interference of foreign substances in complex materials and to obtain greater specificity in the methods, other than that of Sullivan, the specificity of which is still to be regarded as phenomenal.

Rossouw & Wilken-Jorden (47), in a study of the effects upon the Sullivan method of certain variations in the procedure and of a large number of interfering substances such as salts and amino acids, concluded that in hydrolysates of complex materials it would be necessary to isolate the cystine previous to its determination and, furthermore, that the Sullivan method gives more constant results if the concentration of sodium cyanide is doubled and if the time interval between the additions of the color reagent and the sodium sulfite is carefully regulated. They then elaborated a cuprous mercaptide method (48) for the separation of cystine from protein hydrolysates preliminary to the application of their modified Sullivan determination. The method, a modification of one introduced by Vickery & White (49), precipitates the cystine as insoluble cysteine cuprous mercaptide by the direct addition of a solution of cuprous chloride in potassium chloride to the hydrolysate without the preliminary reduction of the cystine to cysteine. Brand, Cahill & Block (50), in examining the urine of cystinuric individuals under various experimental conditions, reported that homocystine depresses the color reaction

produced by cystine in the Sullivan reaction, and that ascorbic acid also probably interferes with the color intensity. Hess & Sullivan (51) have reported that they encountered no difficulty in the determination of either cystine or cysteine in butyl alcohol extracts from normal hydrochloric acid solutions. Lugg's modification of the Folin-Marenzi method was revised by Kassell (52) in order to develop a procedure for the determination of cystine and related disulfides with the Pulfrich photometer. In this study it was found that homologous disulfide compounds such as homocystine, pentocystine, and hexocystine showed a rate of color development which decreased with increasing molecular weight. It was reported that the rate of color development may be accelerated by increasing the amount of sulfite, thus permitting the determination of any one of these disulfides in the presence of another. The interfering effects of various substances upon the Folin-Marenzi procedure have been investigated thoroughly by Shinohara (53, 54). It was also noted in this study that the complete reduction of cystine by zinc even in strong acid and at high temperatures took more than twenty-four hours, and the reduction of cystine by tin, except in powder form, was also slow. Shinohara (54) claims that Lugg's use of mercuric chloride to determine extraneous reducers (55) is not suitable but that formaldehyde, since it does not produce color with the reagent but reacts with cysteine in the reaction mixture, can be used in the Folin-Marenzi method for the determination of the oxidized form, in a mixture of cystine and cysteine or of dithiodiglycolic and thioglycolic acids. In establishing a precision method for the determination of cysteine Shinohara (56) obtained evidence that two reactions of different velocities occur when cysteine or thioglycolic acid is mixed with mercuric chloride in a medium of pH about 5, resulting in the production of two types of compounds having the compositions $\text{Hg}(\text{SRCO}_2\text{H})_2$ and $\text{Hg}(\text{SRCO}_2\text{H})(\text{SRCO}_2\text{HgCl})$. The former corresponds with one of the two structures reported by Claesson in 1877 and by Audreasch in 1879 as resulting from the reaction of thioglycolic acid and mercury compounds.

A modification of the nitroprusside test for sulfhydryl compounds which increases significantly the usefulness of this method has been described by Giroud & Bulliard (57). Zinc acetate is used to stabilize the red color and the reaction is carried out at a neutral pH. Joyet-Lavergne, also, has employed the method for the identification of the sulfhydryl group in tissue slices (58). Fujita & Iwatake (59) have

described a quantitative sodium nitroprusside procedure in the determination of true glutathione from tissues, employing metaphosphoric acid as a protein precipitant. Interference in the determination by any free cysteine which might result from autolysis of the tissues is avoided by immediate freezing of the freshly removed organs.

Studies of the indirect iodimetric determination of cysteine have been described by Lavine (60). The results indicate that its oxidation to cystine may obtain at room temperature if excess of iodine is avoided, if the time interval before titration is short, and if the final concentrations of hydrochloric acid and potassium iodide are molar. Quensel & Wachholder (61) have examined, step by step, the accuracy of the Woodward & Fry titration method (62) in the determination of oxidized and reduced glutathione in tissues and have concluded that the use of sulfosalicylic acid as a protein precipitant affords a more complete extraction of the glutathione than do trichloroacetic or tungstic acids and that the method of extraction, the use of zinc for reduction of SS to SH, and the method of titration could be used for both macro- and micro-analysis of tissues. The use of the iodimetric titration for the determination of diethylsulfide, as proposed by Christomanos (63), has been criticized by Medes, Evangelides & Shinohara (64) since they have found that the removal of iodine from solution by diethylsulfide is probably due to the solubility of the iodine in the sulfide and does not take place stoichiometrically to give $(C_2H_5)_2SI_2$. Woodward's (65) utilization of glyoxalase as a specific reagent for the quantitative micro-estimation of glutathione is unique in that the method is based upon the principle of enzyme-activation specificity.

A comparison of the quantitative results of the oxidation of the sulfur of proteins with different reagents has been used by Blumenthal & Clarke (66) as a means of differentiating between the various forms of sulfur in proteins. The method is based on the observations that the sulfur of methionine, unlike that of cystine, is resistant to oxidation to sulfate by fuming nitric acid and to the production of lead sulfide by the usual procedure, and to the fact that the sulfur of the thiolimidazoles, unlike that of cystine and methionine, is oxidizable to sulfate with bromine. Preliminary studies were made of the labilities of the sulfur of many organic compounds, and of a variety of proteins. From these studies they concluded that methionine was present in all of the proteins which were examined with the exception of the keratins, and that the latter, along with a number of other pro-

teins including zein, contained at least two sulfur-containing constituents in addition to cystine and methionine. The demonstration of the presence in some of these proteins of sulfur oxidizable to sulfate by bromine suggested that thiolimidazoles, presumably thiolhistidine, may occur as natural constituents of the protein molecule.

Mirsky & Anson (67) have now presented the complete report, following their preliminary note published some years ago, of a direct and an indirect colorimetric determination for the estimation of sulfhydryl groups in proteins. The direct method is based on the oxidation of sulfhydryl by a measured excess of cystine. The indirect method employs a preliminary treatment with such an agent as iodoacetate for removal of the sulfhydryl groups and then, after removal of the reagent, the protein is hydrolyzed. The total cysteine content is then determined and compared with that of the untreated protein. A procedure was also elaborated for the determination of the disulfide groups of proteins. The principle of the specific reduction of the protein disulfide by cysteine upon which this method is based is of considerable importance since it has afforded the basis for various studies on the reduction of insulin and other hormones.

Cystine content of tissues and of isolated proteins.—The various methods of analysis for sulfur compounds have been of value for determining the distribution of these substances in isolated proteins and in tissues. Wilkerson (68) reported the content of cystine in the stratum corneum of human skin to be 2.31 per cent as determined by the Folin-Marenzi method. He also observed a ratio of histidine : lysine : arginine in the keratin-like fraction which he claimed was in good agreement with the values found by Block & Vickery (69) for other human keratins such as those of hair and fingernails. Eckstein (70) and Block (71), on the other hand, used the same colorimetric method for the determination of cystine and reported values of 3.82 and 3.40, respectively, for the percentage content of the insoluble non-protease-digestible protein fractions of human skin, and found the molecular ratio of histidine : lysine : arginine to be of the order of 1:6:7, figures which do not agree with the ratio of 1:4:12 which Block (72) has concluded is the approximate proportion of these particular amino acids in all true keratins. Calvery & Freyberg (73) have reported the results of analyses of samples of Bence-Jones protein. These studies have been made in an effort to determine whether these proteins, when excreted by different individuals, are identical compounds, and also whether identical compounds are ex-

creted by the same individual at different times. For determination of the cystine content the Folin-Marenzi method was used; in samples obtained at different periods from the same individual the percentage concentration of this amino acid in the ash-free moisture-free protein was found to be 3 per cent. Csonka (74) has determined and tabulated the high percentages of the nutritionally essential amino acids present in protein preparations from yeasts. The cystine contents, determined after special hydrolysis by a modified Sullivan reaction, were found to be 0.3 per cent for brewers' and 0.27 per cent for bakers' yeast when calculated on moisture-free yeast. These values are considerably lower than those of 0.92 and 0.52 per cent for brewers' yeast as reported by Prunty (75). The percentages of cystine, tryptophane, and of tyrosine were found to vary widely in different varieties of the soy bean according to Csonka & Jones (76), who suggested that varieties of the bean should be selected for planting which produce not only greater amounts of protein but also better quality from the nutritional standpoint.

Glutathione content of blood and tissues.—In an effort to throw some light on the physiological functions of glutathione much work is now beginning to appear on the variations in the concentration of glutathione in the blood and tissues under a variety of conditions. Definite conclusions, however, cannot be drawn from the work as yet but, out of the numerous studies, certain generalizations seem to be taking form. In the first place, it appears that the glutathione content of more active muscles is higher than that of less active (77, 78); secondly, that prolonged physical training leads to an increase in the glutathione content of muscles (79), and, thirdly, that there is an indication that the glutathione of the blood is decreased during pregnancy (80, 81). Space does not permit reviewing the many papers along these lines but before leaving this phase of the work, we should like to call attention to the interesting observations of Oberst & Woods (82, 83) who found no apparent correlation between the percentage of oxygen saturation and the percentage of reduced or oxidized glutathione in human venous blood and observed that the disappearance of glutathione upon incubation of whole blood depends upon diffusion of the glutathione from the cells where it is normally present, into the plasma where it is inactivated or destroyed through chemical reaction with the plasma protein. It will be interesting to see what further work will bring out as to the significance of this rather surprising finding.

Relation of dietary constituents to tissue composition.—In an important contribution to this problem, Lee & Lewis (84) were unable to support the views of Schenck & Wollschitt (85) that the tissue proteins themselves have a composition dependent upon the diet, for they found that the composition of kidney and muscle tissue of young and adult rats was not affected by a fast of from forty-eight to seventy-two hours. In the case of liver tissue, both total nitrogen and total sulfur were considerably increased after fasting, but it was not possible to correlate the increases with any changes in the composition of the liver proteins. The sulfur content of the liver, muscle, and kidney was also higher in the animals receiving an adequate cystine supplement than in those on a cystine-deficient diet, but the extra sulfur was again due to some non-protein sulfur compound in the alcohol-water extract obtained after the separation of the protein. Pertinent in some respects to these observations are those of Marenzi & Braier (86) who found a decrease in the total glutathione content of various tissues of rats as a result of maintenance on a cystine-deficient diet, and the experiments of Binet & Weller (87) which also demonstrated that in guinea pigs there occurred no significant changes in the glutathione of the kidneys, muscles, and a great many other tissues during a starvation period of eight days.

Potter & Franke (88) have presented studies of the effect of various diets upon the concentration of ergothioneine in the blood of rats. They concluded that this constituent is entirely exogenous in origin.

Absorption and oxidation studies, in vivo.—Measurements of the absorption and the oxidation in the animal organism of a considerable number of sulfur compounds have been carried out in various laboratories. Andrews, Johnston & Andrews (89), using a dog with isolated intestinal loops, found the following relative rates of absorption for a series of compounds in the order of the speed of absorption: cysteic acid > cysteic-acid-hydantoin > cystine-phenylhydantoin > dibenzoylcystine > cystine > cystinehydantoin. Oxidation data from the sulfur partition of the urine after the administration of these substances confirmed the results of previous studies on unoperated animals in the case of cystine, cysteic acid, cystinephenylhydantoin, and dibenzoylcystine. Stekol (90) found that *dl*-methionine was retained as well as *l*-methionine by adult dogs on a protein-free diet and by growing dogs on a low sulfur diet. This observation was quite different from the results of his studies with *dl*- and *l*-cystine in

which the racemic form was not as well retained, indicating that *d*-cystine was not utilizable like *l*-cystine (91). These results agree with those found in the studies on utilization of the compounds for growth purposes which have demonstrated that both *d*- and *l*-methionine are utilized by the animal organism whereas only the naturally-occurring isomer of cystine is utilized (92, 93). In this connection the observations of Bernheim & Bernheim (94) should be considered, which indicate that purified enzyme preparations from the kidneys of various mammals preferentially oxidize the foreign isomers of a number of amino acids, including cystine. These investigators mentioned the work of Stekol (91) as indicating that *d*-cystine was more readily oxidized than *l*-cystine. Without detracting from the general significance of the interesting results of Bernheim & Bernheim, it should be pointed out that Stekol's experiments had no bearing on this particular point. Stekol's interpretation of his own results was that under those particular experimental conditions the *l*-cystine which the animal was in need of was retained in the body whereas the *d*-cystine, not being utilizable for this purpose, was oxidized and the sulfur excreted as sulfate. Stekol's results cannot be construed as indicating that the body as a whole can oxidize *d*-cystine more readily or more completely than *l*-cystine. In the opinion of the reviewers, comparative studies on the oxidation of sulfur compounds in the body must be carried out with the animals on a diet supplying sufficient *l*-cystine for body functions, otherwise one would encounter retention of *l*-cystine as experienced by Stekol.

Growth experiments with sulfur-containing compounds.—The application of the essential nature of certain amino acids in growth studies has continued to provide one of the most valuable methods available for throwing light on the very difficult question of the intermediary steps involved in the metabolic breakdown of these amino acids in the body. Such studies have been of great aid in the investigations of sulfur compounds and have brought out the close metabolic relationship between methionine, homocystine, and cystine. The method has also been of aid in indicating whether a given derivative of an amino acid is hydrolyzable in the body by feeding the derivative to animals on a diet deficient in that particular amino acid. Such a study has been made by Jones, Andrews & Andrews (95) on a series of cystine derivatives and the results have indicated that cystine-hydantoin, and cystine-phenylhydantoin are not hydrolyzed *in vivo* to cystine whereas dibenzoylcystine is slightly hydrolyzed. The latter observation

agrees with previous results of Lewis *et al.* (96) which demonstrated that this compound is partially hydrolyzed when it is administered *per os*. Lewis and coworkers found that when the dibenzoylcystine is injected it is not hydrolyzed. In this connection it may be noted that unpublished experiments of the writers of this review have demonstrated that acetyl-*l*-cystine is as well utilized for growth purposes when injected intraperitoneally into rats as when it is fed, indicating that the tissues can remove the acetyl group, a behavior which is in contrast to the failure to remove the benzoyl group.

Studies of the utilization of the optical isomers of homocystine have demonstrated that both *d*- and *l*-homocystine are utilizable for growth purposes by animals on a cystine-deficient diet (97). This physiological behavior of homocystine with respect to spatial configuration is therefore similar to that of methionine but in contrast to that of cystine. This observation further supports the idea that has been suggested (12, 98, 99) that homocystine may be physiologically concerned in the metabolic relationship which appears to exist between cystine and methionine, since, if methionine should be converted to homocystine in the body, one would expect that *d*- and *l*-homocystines, like the corresponding *d*- and *l*-methionines, would be physiologically active.

Mitchell (100) has reported that he has been unable to obtain by his paired-feeding method any evidence to support the observation of Sullivan, Hess & Sebrell (101) that dithioethylamine (cystine amine) can "replace cystine to a considerable degree for the purpose of growth of the young white rat."

The effects of cystine deficiency upon the utilization of energy-producing nutriment and protein have also been studied in the rat by Swift, Kahlenberg, Voris & Forbes (102). This constitutes the first example of such a study of the effects produced by individual nutrient deficiencies. The animals with the cystine supplements made greater growth and stored 10.8 per cent more energy and 24.4 per cent more nitrogen than did the control animals.

Cystinuria.—The unpublished experiments on methionine feeding to cystinurics, which were mentioned by Lewis¹ as confirming the observations of Brand and his coworkers, have now been reported (103). It is rather interesting that in this work of Lewis it was noted that the excretion of cystine after the ingestion of *dl*-methionine was greater

¹ *Ann. Rev. Biochem.*, 4, 163 (1935).

and extended over a longer period of time when the diet contained moderate amounts of protein than when it contained large quantities of protein. While, in general, Lewis' observations confirm the work reported earlier by Brand & Cahill (104) with methionine, Andrews & Randall (105) found that no significant increase in urinary cystine followed the ingestion of 2.0 to 5.0 gm. of *dl*-methionine by a fourteen-year-old cystinuric. They suggest that considerable variation may exist between cystinuric subjects. Brand and his colleagues (106) have published the details of their work, a preliminary report of which was reviewed last year. They have offered additional evidence which they feel supports their early view that "the fundamental difficulty in the cystinuric individual arises not from an inability to handle the cystine but rather the methionine in the food and also the cysteine which may be formed during protein digestion." They go further to assume that "although the cystine excretion in cystinuria is caused mainly by dietary methionine, the inborn error of metabolism is concerned with the handling of cysteine." They have interpreted their experiments as indicating that there are probably separate mechanisms for the oxidation of $-SS-$ and $-SH$ compounds and for the same compounds separately or in peptide form. Their experimental observations in the cystinuric (50, 106) upon which these views are based are the following: cystine, homocystine, and glutathione-SH are almost completely oxidized, the first two substances causing no increase in urinary cystine excretion and glutathione yielding only a small amount of extra cystine; on the other hand cysteine, homocystine, and methionine are excreted largely as extra cystine, only a small part being oxidized to inorganic sulfate. From their results on methionine and homocystine in the cystinuric, Brand and collaborators have come to the conclusion that demethylation of methionine may be the first major step in the catabolism of methionine by way of cysteine, an observation which confirms the theory of demethylation of methionine discussed by others. Because of the close structural relationship between cysteine and serine, Brand & Cahill (107) studied the effect of the oral administration of *dl*-serine alone and of a mixture of *dl*-serine and *l*-cystine upon the daily excretion of cystine by a cystinuric subject. In neither instance was an effect upon the urinary cystine excretion noted.

The question has naturally arisen as to whether the various tissues, particularly the hair and nails of a cystinuric, have a decreased cystine content. Lewis (103) found that the cystine content of the hair of three cystinuric children, five to ten years of age, did not differ ma-

terially from that of their normal brothers and sisters of approximately the same age; analyses of the hair of adult cystinurics also showed no abnormalities in cystine content. Lewis & Frayser (108) have also confirmed the finding of Sullivan & Hess that the nails of cystinurics were normal in cystine content. In discussing this failure of the loss of cystine by the kidney to influence the cystine content of these tissues, Lewis & Frayser point out the recent observations that epidermal tissues are low in methionine, and that methionine may be the source of cystine in the urine. The cystine of the tissues would not necessarily be expected to be affected if cystinuria does not involve an error in cystine utilization.

Hickmans & Smallwood (109) have made some very thorough studies on two cystinuric sisters which have confirmed much of the work of other investigators with respect to the complete oxidation of cystine administered in the free state, the increase in the Sullivan value of cystinuric urine upon standing, and the increase of cystine excretion upon the feeding of protein, particularly of animal protein.

An almost unique case of cystinuria associated with a cystine calculus in a dog has been described by Morris, Green, Dinkel & Brand (110). It was indicated that attempts will be made to breed a cystinuric strain of dogs. This finding may play an important rôle in the studies of sulfur compounds even as the observation that the Dalmatian hound excreted uric acid contributed to the metabolic studies on uric acid.

Mercapturic acid synthesis.—White & Jackson (111) have compared the effects of taurine, sodium sulfate, and an amino acid digest prepared from casein with the effects of *l*-cystine and of methionine in alleviating the cystine deficiency produced by the incorporation of brombenzene in the diet of rats. Since taurine, sodium sulfate, and the digest were ineffective in stimulating growth, it was concluded that cystine and methionine are specific in this respect.

The observation made by Bourne & Young (112) with rabbits has been made also with adult and growing dogs by Stekol (113) who has isolated *l*- α -naphthalene mercapturic acid from the urine following the oral administration of naphthalene. The neutral sulfur of the urine was also found to be increased by naphthalene ingestion, and the formation of ethereal sulfate was indicated. Qualitative tests also indicated an increased output of glycuronic acid.

Stekol (114) has also fed brombenzene to growing dogs and mice on adequate diets and to fasting growing dogs; *p*-bromphenylmercapturic acid was isolated from the urine in all cases. Apparently the dogs

that were fasting were capable of supplying cystine for detoxication purposes at the expense of tissue.

Rôle of sulfur compounds in cellular oxidation-reduction.—While the discussions of cellular oxidation-reduction reactions have centered about the glutathione-SH \rightleftharpoons glutathione-SS- system for a long period since the properties of the tripeptide were described by Hopkins (115), the literature at present reflects a widening consideration of the factors involved. Included in the consideration of this question are not only glutathione and cytochrome but also the respiration enzyme of Warburg, ascorbic acid, adrenaline, the flavins, and vitamin A. Space does not permit a review of the various researches on the interrelationship of these factors with sulfur compounds but mention might be made of the stimulating hypothesis of Joyet-Lavergne (58) which suggests that the mechanism of the catalysis of intracellular oxidation-reduction is effected through the medium of the chondriomite by the collaboration of two of its constituents, glutathione and vitamin A.

Relation of -SH and -SS- to cellular growth and cancer.—Additional experimental observations have been reported concerning Hammett's theory (116) that the sulfhydryl group is a specific stimulus for cellular growth but the controversial status of the general problem still obtains (117-120). A broad survey of this field may be found in the *Cold Spring Harbor Symposia* (121). While the discovery of the carcinogenic effect of certain cyclic hydrocarbons seems to have diverted to some extent the attention of investigators from a possible relationship between sulfur metabolism and malignancy, this question has received some consideration within the past year. Comparisons of the sulfur content of normal with that of corresponding malignant tissues have been made (122-125) and the effects of cystine-poor and cystine-rich diets upon the growth of transplanted tumors have been investigated (122, 126). In addition, fundamental researches bearing on the effects of oxygen tension on protein synthesis and proteolysis with reference to the sulfhydryl-disulfide system and enzyme action have been undertaken (127, 128).

Growth of hair and wool.—The recent investigations of the effect of dietary sulfur upon the growth of hair and wool have not changed to any degree the outlook of that problem since it was reviewed last year. For this reason no discussion is included here and only a few references are given to later papers which report the results of the application of improved analytical methods to the question of the sulfur composition of pasture grasses and of wool, and the effect upon the

composition and quantity of wool and hair of various sulfur supplements to controlled diets (48, 129-134).

Relation of sulfur to vitamin-B₂ deficiency.—With respect to the physiological aspects of the vitamin problem it is evident that there is renewed interest in the observation which has been made frequently in the past, both in clinical and in experimental investigations, of some sort of a relation between a deficiency in the vitamin-B₂ complex and a disturbed sulfur metabolism.

A direct reference to a possible relationship between vitamin-B₂ deficiency and sulfhydryl deficiency has been made by Itter, Orent & McCollum (135) who observed a curative action of a supplement of cysteine or glutathione to a vitamin B₂-deficient diet on the alopecia accompanying B₂-deficiency in rats, and a beneficial effect on the maintenance of weight. These investigators interpreted their experimental observations in the light of other work of similar nature as evidence that vitamin-B₂ deficiency may frequently be complicated by a superimposed lack of substances possessing the sulfhydryl group.

Relationship of sulfur compounds to micro-organisms.—Experimental observations concerning the biochemistry of sulfur compounds in connection with the metabolic behavior of micro-organisms have been increasing but the work is at present in its pioneering stage and it would be difficult to attempt to draw any definite conclusions. The studies of Mueller (136) on the cultural needs of a special strain of diphtheria bacillus have indicated that there may be a possible synergistic effect of methionine and histidine under special experimental conditions. Tarr (137), in additional studies of the metabolism of sulfur compounds in *Proteus vulgaris* and *Serratia marcescens*, has obtained evidence of the existence in the cells of these organisms of a special enzyme for the formation of hydrogen sulfide from organic sulfur compounds, and of the requirement of an amino group for the activity of the enzyme. The rate of oxidation of *dl*-methionine, among other amino acids, by "resting" *Bacillus proteus* has been measured by Bernheim, Bernheim & Webster (138). Of interest in connection with the mechanism of the action of hemolysins is the work of Cohen & Shwachman (139) which has demonstrated a reversible inactivation of pneumococcal hemolysin by various treatments which would be expected to oxidize any sulfhydryl groups to disulfide, or in some way to block the existing thiol groups. The behavior was essentially similar to that of urease and papain under the same experimental conditions. As another type of investigation of the sulfur problem in micro-

organisms may be mentioned the finding of 3.1 per cent of cystine in the protein of type II pneumococcus by Calvery, Heidelberger & Kendall (140), and the observation made by Lawrie (141) of the probable presence of glutathione in the protozoon *Glaucoma pyriformis*.

Selenium and sulfur.—Because of the close chemical relationship of the element selenium to sulfur, it may not be out of place to call attention here to the very interesting problem which has arisen as a result of the tracing by Franke *et al.* (142) of a disease of animals known as "alkali disease" to the crops grown in certain localized soil areas. As an outgrowth of this discovery, investigations were undertaken by several bureaus in the United States Department of Agriculture and the South Dakota Agricultural Experiment Station which have disclosed that the vegetation in question contains selenium in organic combination in the protein fractions (143–148). The interesting possibility has arisen that there may occur selenium analogues of the sulfur-containing amino acids. Further relationship between the metabolism of sulfur and selenium in plants is strikingly shown by the work of Hurd-Karrer (144) in which an antagonism between these elements has been demonstrated.

Reviews.—An excellent review of the behavior of the chief sulfur compounds in nutrition has been made by Lewis (149). A review of certain phases of the chemistry of organic sulfur compounds by Gibson might also be mentioned (150).

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DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF MEDICINE, GEORGE WASHINGTON UNIVERSITY
WASHINGTON, D.C.

CHEMISTRY AND METABOLISM OF COMPOUNDS OF PHOSPHORUS*

BY ROBERT ROBISON

The Lister Institute of Preventive Medicine, University of London

During the last few years a very notable advance has been made in knowledge of the part played by phosphoric acid in carbohydrate metabolism¹ concerning which the first clear evidence was furnished by Harden more than thirty years ago. A large part of this review is devoted to the consideration of these new discoveries whose significance extends beyond the limits of their immediate application.

Reference may first be made to the account of Embden's share in this progress as told by his coworkers (1). Embden's new scheme of lactic acid fermentation in muscle extracts appeared in 1933 (2) and was adopted by Meyerhof, who with his coworkers (3) provided additional evidence in its support and proposed an analogous scheme of alcoholic fermentation in yeast juice (4). Both schemes began with the conversion of hexosediphosphate into triosephosphate which, by an oxidation-reduction process, yields α -phosphoglycerol plus 3-phosphoglyceric acid; phosphoglyceric acid breaks down to pyruvic and phosphoric acids. In muscle extract, pyruvic acid is reduced to lactic acid at the expense of the phosphoglycerol which is oxidised to triosephosphate. In yeast juice, pyruvic acid is converted by carboxylase into carbon dioxide plus acetaldehyde. The latter then takes part with glucose and inorganic phosphate in a rapid reaction in which hexosediphosphate plays the part of a catalyst and phosphorylation is coupled with an oxidation-reduction; the primary esterification product is oxidised to phosphoglyceric acid while the acetaldehyde is reduced to alcohol. Meyerhof supposes the primary ester to be an unstable compound differing from the known hexose- or triose-phosphates. This reaction (*vide* scheme of alcoholic fermentation, p. 187, equation D) characterises the "stationary phase" of rapid fermentation. In the initial phase, before acetaldehyde has been produced, the reaction represented by equation A occurs; hexosediphosphate, which alone is very slowly fermented by yeast juice, breaks down

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¹ Cf. also this volume, pp. 1, 31. (EDITOR.)

more rapidly in presence of glucose and inorganic phosphate, equivalent quantities being simultaneously esterified and converted into phosphoglycerol and phosphoglyceric acid, probably by way of triose-phosphate.

The knowledge gained during the last two years has led to considerable modifications in both schemes. Meyerhof at first considered the triosephosphate to be phosphoglyceraldehyde, which had been synthesised by Fischer & Baer (5) and possessed the required biological properties. Meyerhof & Lohmann (6) then isolated the triose ester formed by the action of dialysed muscle or yeast extracts on hexosediphosphate; this ester, or at least its major constituent, proved to be phosphodihydroxyacetone. It was shown (7) that in such dialysed extracts an equilibrium mixture of hexosediphosphate and phosphodihydroxyacetone is very rapidly formed from either ester. The enzyme, zymohexase, which catalyses this reaction is relatively thermostable and is not inhibited by fluoride, oxalate, or iodoacetate. The equilibrium was shown to obey the equation

$$K_T = [\text{phosphodihydroxyacetone}]^2 / [\text{hexosediphosphate}]$$

the value of K being lowered by magnesium ions and very markedly influenced by temperature. The enzymic splitting of hexosediphosphate into two mols of phosphodihydroxyacetone is an endothermic process; the heat of reaction was found experimentally to be $-14,000$ calories per mol of hexosediphosphate, a value in fair agreement with that ($-12,000$ calories) calculated from the van't Hoff equation, $-U = RT^2 \text{dlog } K/\text{dT}$ (8). The identity of the triose ester was confirmed by Kiessling's (9) synthesis of phosphodihydroxyacetone. Like phosphoglyceraldehyde it is decomposed in a few minutes by N NaOH at room temperatures, yielding lactic and phosphoric acids, while in N HCl at 100° methylglyoxal and phosphoric acid are formed. It is not oxidised by hypoiodite and is thus distinguished from phosphoglyceraldehyde. These findings do not exclude the possibility that phosphoglyceraldehyde is also formed, as postulated in Embden's scheme (1, 2). This seems more probable since Meyerhof & Kiessling (10) have now shown that in dialysed muscle extracts phosphoglyceraldehyde is very rapidly converted into phosphodihydroxyacetone; an equilibrium between the two esters may exist though proof of this is not yet forthcoming.

Much additional light has been thrown by Meyerhof and his

colleagues on the conversion of (—)3-phosphoglyceric acid into pyruvic and phosphoric acids; this has been shown to take place in three stages. Two new phosphoric esters, formed as intermediate products, have been isolated: phospho-enol-pyruvic acid by Lohmann & Meyerhof (11) and (+)2-phosphoglyceric acid, ($[\alpha]^{20}_D$, +24.3°) by Meyerhof & Kiessling (12). In dialysed extracts of muscle or yeast, 3-phosphoglyceric acid is converted into an equilibrium mixture of the three esters by successive reversible reactions, (—)3-phosphoglyceric acid \rightleftharpoons (+)2-phosphoglyceric acid \rightleftharpoons phospho-enol-pyruvic acid, catalysed by two enzymes which have been partially separated from one another and named phosphoglyceromutase and enolase respectively (13, 14). Coenzyme is not required for these reactions but is essential for the third stage, the formation of pyruvic acid, which is irreversible. Sodium fluoride inhibits chiefly the second reversible reaction. The attainment of these results was greatly aided by Kiessling's synthesis of phospho-enol-pyruvic acid (15) and of the racemic 3-phosphoglyceric and 2-phosphoglyceric acids (16). Both the latter were fermentable to the extent of 50 per cent (one optically active component) by yeast juice; the residual, biologically inactive, (+)3-phospho- and (—)2-phosphoglyceric acids were isolated and had $[\alpha]^{20}_D$, +14.5° and —23.5° respectively (12).

It is an essential requirement of any scheme that all intermediate reactions should be capable of proceeding with a velocity at least as great as that of the whole process. Not all the reactions of the Embden-Meyerhof schemes satisfied this requirement. In muscle extract the reaction between pyruvic acid and phosphoglycerol takes place (3) but usually at a very much slower rate than that at which lactic acid is produced from carbohydrate or hexosediphosphate (17, 18). In yeast juice, pyruvic acid is decomposed by carboxylase even more rapidly than the maximum rate of fermentation of sugar, yet phosphoglyceric and phosphopyruvic acids, the immediate precursors of pyruvic acid, are fermented at only one-tenth this rate (19). Further, Meyerhof & Kiessling's first scheme of alcoholic fermentation (4, 19) offered no explanation of the equivalence between phosphate esterified and carbon dioxide produced during the rapid phase of fermentation, i.e. the reaction expressed by Harden & Young's first equation (20). The difficulties first enumerated have been overcome by the discovery of new reactions involving the transference of phosphoric acid from molecule to molecule by the agency of the co-

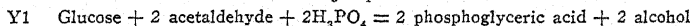
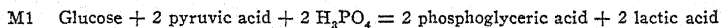
enzyme; these reactions have, at the same time, provided a possible explanation of Harden's equation. Meyerhof & Lohmann (21) were the first to suggest that the rôle of adenosinetriphosphate as coenzyme in muscle glycolysis consists in the initial esterification of carbohydrate by the labile phosphoric acid groups, the triphosphate being resynthesised at a later stage in the carbohydrate breakdown. This conception of the coenzyme as a phosphate carrier is now supported by the clearest evidence. It was shown by Lohmann (22) that the splitting of phosphocreatine in dialysed muscle extracts takes place only in the presence of adenylic acid which is simultaneously converted into adenosinetriphosphate. Earlier experiments of Meyerhof and Lohmann had suggested that the reverse reaction, phosphorylation of creatine by adenosinetriphosphate, also takes place and this has been confirmed by Meyerhof & Lehmann (23, 24) and by Needham & van Heyningen (25).

The investigations of Parnas and his coworkers (26) on the formation of ammonia in muscle led to the discovery of another important reaction. It had been shown that muscle deaminase, which liberates ammonia from adenylic acid, does not attack adenosinetriphosphate. In muscle pulp, ground with water or phosphate solution, formation of ammonia was delayed; but addition of iodoacetate or fluoride caused the immediate liberation of ammonia, from which it was inferred that the continuance of glycolysis is necessary for the maintenance of adenosinetriphosphate. It was shown (27) that the liberation of ammonia in the presence of iodoacetate could be prevented by the addition of phosphoglyceric or phosphopyruvic acid or by pyruvic acid plus inorganic phosphate. This effect was at first attributed to the transference of phosphoric acid from phosphoglyceric acid to creatine and thence by Lohmann's reaction to adenylic acid. The formation of phosphocreatine under such conditions was verified but subsequent investigations (23, 24, 25, 28) proved that phosphoric acid is transferred from phosphopyruvic acid to adenylic acid, yielding adenosinetriphosphate, and thence to creatine. The phosphorylation of adenylic acid proceeds equally well in absence of creatine, while that of creatine does not take place unless adenosinetriphosphate (or adenylic acid + phosphopyruvic acid) is present.

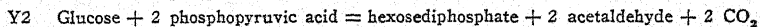
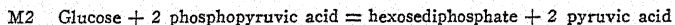
Another discovery bearing on the question of phosphate transfer was made by Lipmann (29) in the course of his experiments on potential variations in yeast maceration juice during fermentation. The period of induction before fermentation begins in such juice is

abolished or greatly shortened by addition of very small amounts of hexosediphosphate (20). Lipmann found that phosphoglyceric acid had an even more pronounced effect and, correlating this fact with the findings of Lohmann and Parnas, concluded that the phosphate group must be transferred with great ease from phosphoglyceric acid to glucose.

This leads to the consideration of Meyerhof's most recent papers (17, 30, 31) describing new reactions which occur in yeast and muscle extracts and bring into still closer accord the two processes of carbohydrate breakdown: (a) In muscle extract containing sodium fluoride (which inhibits the fermentation of carbohydrate), pyruvic acid reacts very rapidly with inorganic phosphate and glucose in presence of yeast hexokinase according to equation M1. With glycogen a similar reaction takes place without hexokinase. M1 is the counterpart of the previously known reaction Y1 which occurs in yeast maceration juice. Both reactions are inhibited by iodoacetate.

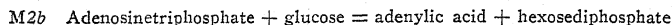
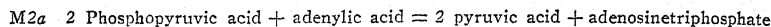


(b) Phosphopyruvic acid and glucose take part in a reaction in which the phosphoric acid is transferred to the hexose molecule, the rate of the reaction being many times greater than that at which phosphopyruvic acid breaks down in absence of glucose. In presence of iodoacetate the reactions in muscle extract and in yeast juice are represented by equations M2 and Y2. If iodoacetate is replaced by fluoride and inorganic phosphate is present, the pyruvic acid or acetaldehyde reacts further according to equation M1 or Y1.

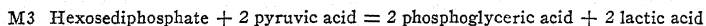


"Hexosediphosphate" here represents a mixture of hexosemonophosphate, hexosediphosphate, and triosephosphate. In yeast juice, phosphate is transferred with equal rapidity to glucose, fructose, or mannose; somewhat more slowly to hexosemonophosphate and still more slowly to glycogen, starch, galactose and other carbohydrates. The velocities of all these reactions are at least equal to those of the whole fermentation processes in absence of inhibitors. All require the presence of coenzyme. In muscle extract (with hexokinase) the

intermediate reactions M2a and M2b have been quantitatively demonstrated (17):



It cannot as yet be so definitely stated that adenylic acid is the phosphate carrier in yeast juice. Meyerhof considers this to be possible, while Parnas *et al.* (32) are more emphatic; but some caution may be observed until the ability of cozymase as well as of adenylic acid to function as phosphate carrier has been quantitatively investigated (33, 34). (c) Lastly, in muscle extract, but only to a limited extent in yeast juice, hexosediphosphate reacts with pyruvic acid in the presence of sodium fluoride according to equation M3. This reaction, which involves no transfer of phosphoric acid, takes place in dialysed extracts and does not appear to require participation of adenosinetriphosphate, though it is recognised that a coenzyme, similar to cozymase but bound to a non-dialysable carrier, may be present in the extract:



Quantitatively, the Embden reaction between pyruvic acid and phosphoglycerol in muscle extract seems to play only a small part in the formation of lactic acid.

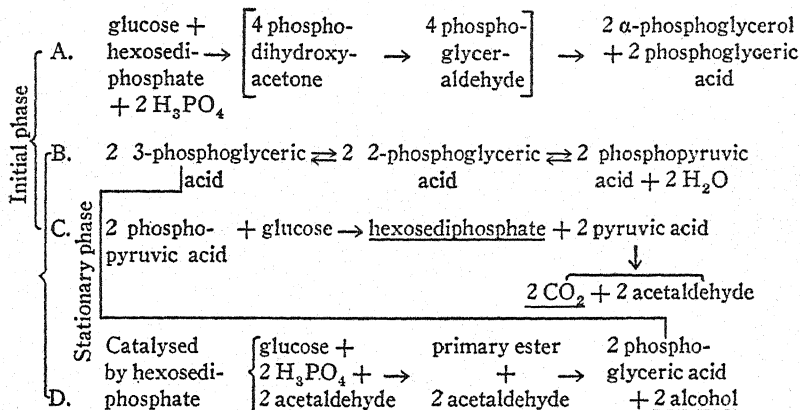
In the light of the above facts Meyerhof & Kiessling (17, 31) have put forward modified schemes of alcoholic and lactic acid fermentations. These are shown below.

The formation of phosphoglyceraldehyde is still hypothetical, while hexosediphosphate includes also the mixed hexosemonophosphates. In the scheme of alcoholic fermentation, apart from the intermediate stages of reaction B, the important addition is reaction C, which clearly provides a possible basis for the Harden-Young ratio between phosphate esterified and carbon dioxide produced. Meyerhof tentatively suggests that, in order to explain the accumulation of hexosediphosphate in yeast juice, a lack of synchronism between the transfer of phosphate to hexose and the reduction of acetaldehyde by the primary ester so formed should be postulated. Objections could be raised against this explanation but need not be stressed here.

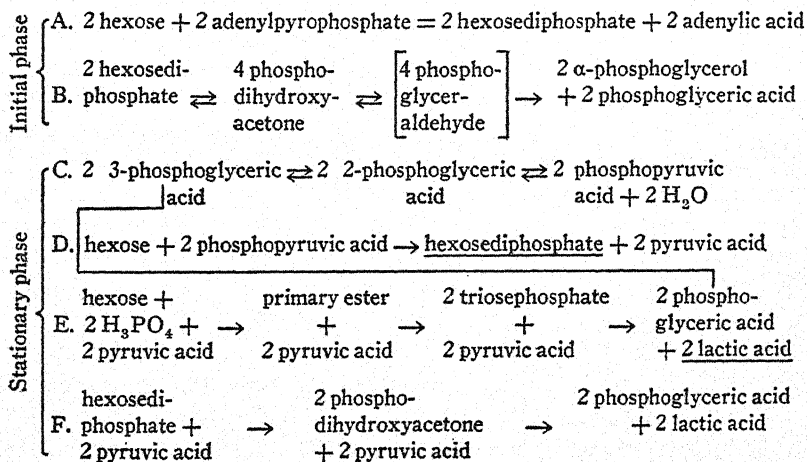
The scheme of the main path of lactic acid formation follows

closely that of alcoholic fermentation. Equations D and E show the equivalence between hexosediphosphate and lactic acid demonstrated by Meyerhof for the glycolysis of sugar with hexokinase in muscle extract.

SCHEME OF ALCOHOLIC FERMENTATION



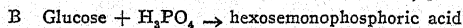
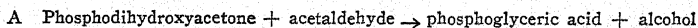
SCHEME OF THE MAIN PATH OF LACTIC ACID FERMENTATION



Among the points which are still not clear are the relationship of hexosemonophosphate to hexosediphosphate in the fermentation

process and the nature of Meyerhof's hypothetical, unstable, primary ester. It is still uncertain to what extent the transference of phosphate and of hydrogen can be effected by different groups in one compound. It is curious that magnesium ions appear to be necessary for some but not for all reactions in which the coenzymes are concerned. There is evidence that esterification of glycogen with inorganic phosphate, forming hexosemonophosphate, may take place both in yeast (35) and muscle (36) extracts without the co-operation of coenzyme, but the mechanism of this process is not clear. Esterification of the hexoses may occur either by transference of phosphate from adenosinetriphosphate or by means of inorganic phosphate, but in the latter case the reaction is always coupled with an oxidation-reduction for which a coenzyme is essential (37).

Information on this coupled reaction has been gained from a variety of investigations. Schöffner *et al.* (38, 39) have attacked the problem by building up a phosphorylating system with purified enzymes. Dialysed glycerol extracts of dried yeast contained an active phosphatase free from dehydrogenases but were unable to phosphorylate glucose even in presence of cozymase, hexosediphosphate, acetaldehyde, and magnesium. Phosphorylation occurred on addition of a redoxase prepared from orange seeds, or of Warburg's intermediate enzyme, to all the above components. It was found that the reaction did not depend on phosphatase but on zymohexase, which was associated with it and could be removed by adsorption on alumina- C_{γ} . Both zymohexase and the intermediate enzyme were purified until no phosphatase activity could be detected. In this purified enzyme system (containing cozymase) the amount of phosphate esterified was equivalent to the hexosediphosphate added. Phosphorylation also occurred in absence of zymohexase when hexosediphosphate was replaced by phosphodihydroxyacetone. The authors concluded that reaction A induces, by some unknown mechanism, reaction B, both being inhibited by iodoacetic acid:



Crude maceration juice contains a mechanism by which hexosemonophosphate is converted into hexosediphosphate, which is thus able to act as a catalyst for these reactions. At about the same time as these results appeared, Runnström *et al.* (40, 41) and Dische (42, 43, 44)

reported experiments showing the esterification of inorganic phosphate in hemolysed red blood corpuscles coupled with the oxidation or degradation of hexosephosphates. Degradation of glucose or hexosemonophosphate was preceded by phosphorylation, at least two-thirds and possibly the whole of the phosphate being derived from adenosinetriphosphate (44).

Euler & Adler (45) have succeeded in isolating from Warburg's intermediate enzyme a specific enzyme "heterophosphatase" which activates the transfer of phosphoric acid from adenosinetriphosphate to hexose, with formation of hexosemonophosphate; it is not, by the usual criteria, a phosphatase. Meyerhof (46) finds that this enzyme is responsible for at least part of the effect of hexokinase, which enables muscle extracts to ferment hexoses. There is evidence that phosphoric acid can be transferred from hexosephosphates to adenylic acid as well as in the reverse direction (47, 48, 49).

Rôle of phosphoric esters in biological oxidations.—Phosphoric esters are intimately concerned in many biological oxidations, as activating coenzymes or as activated substrates. In Warburg's system, hexosemonophosphate (Robison ester) is oxidised by the combined agency of the intermediate enzyme, the hydrogen-transferring coenzyme, the yellow (flavin) enzyme,² and oxygen or methylene blue. The product of this oxidation has been identified as phosphohexonic acid (50). Warburg, Christian & Griesse (50, 51, 52) have purified the coenzyme to a high degree and have been able to throw considerable light on its chemical constitution. The purest preparations yielded on hydrolysis adenine and nicotinamide in proportions which led these authors to conclude that the coenzyme is composed of 1 mol of each of these bases, 2 mols of pentose and 3 mols of phosphoric acid, combined with the loss of 6 mols of water. The analytical data agreed with those calculated for such a compound, $C_{21}H_{28}N_7P_3O_{17}$. Nicotinamide appears to be the hydrogen-transferring component of the coenzyme, though free nicotinamide is quite inactive. In the reversible reduction of the coenzyme by hexosemonophosphate the pyridine ring takes up 2H; the complete reduction of the ring (6H) by hydrogen and platinum is irreversible and renders the substance unable to function in the Warburg system. This coenzyme is widely distributed; it occurs in horse red blood corpuscles to the extent of at least 12 mg. per litre and is present in

² Cf. also this volume, pp. 8, 33, 359. (EDITOR.)

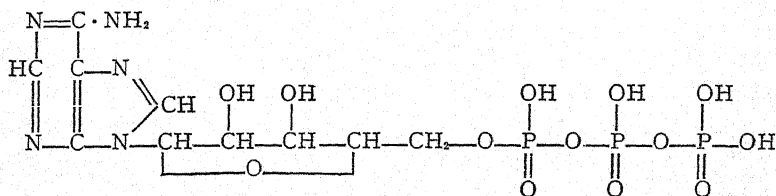
yeast and probably in heart muscle. It is not identical with cozymase although chemically very closely allied to it. Negelein & Haas (53) conclude that the intermediate enzyme is the colloidal carrier of the coenzyme with which it forms a dissociable complex. This complex is the specific dehydrogenase of hexosemonophosphate. The yellow enzyme which takes up hydrogen from the reduced coenzyme and transfers it to oxygen or other acceptor is quite unspecific and is an integral component of many dehydrogenating systems (54, 55). Euler & Adler (45) have shown that addition of adenosinetriphosphate to Warburg's system enables it to oxidise fructose and glucose which are first converted into hexosemonophosphate through the agency of the heterophosphatase. Theorell (56, 57) has been able to effect far-reaching purification of the yellow enzyme by means of cataphoresis, and has described a crystalline form. Dialysis of the purified enzyme against dilute hydrochloric acid produces a reversible scission of the two components, the flavin pigment and the protein carrier, which are separately inactive but on being brought together instantly combine and regain full activity. The flavin component, which had been previously isolated by Warburg & Christian and is to be regarded as the thermostable, dialysable coenzyme of the yellow enzyme complex, has now been identified by Theorell (58) as a flavin monophosphoric ester. Synthetic esters have been prepared (57, 59) by direct phosphorylation of lactoflavin but do not show the enzymic activity of the natural ester. Phosphorylation by the action of intestinal phosphatase is reported by Rudy (60) who suggests that the vitamin-B₂ activity of flavin depends on its phosphorylation in the animal body. Flavin phosphoric acid has been isolated from liver and appears to constitute the major portion of the total flavin in this organ (61).

Cozymase.³—The purification and chemical nature of cozymase have formed the subject of many papers by Euler, Myrbäck and their coworkers. Myrbäck has shown that cozymase is a monobasic acid with strong reducing properties, closely related to adenylic acid, and that it is inactivated by dephosphorylation with kidney phosphatase but is not attacked by muscle deaminase. It is inactivated when heated for two minutes in 0.01*N* NaOH and a second acidic group is then set free (62). After preliminary purification (63) highly active preparations were finally obtained (64) by precipitation as

³ Cf. also this volume, pp. 4, 30. (EDITOR.)

the copper salt and these are believed to be the pure cozymase. Their percentage composition corresponded with the formula $C_{24}H_{35}O_{18}N_8P_2$, while the products of hydrolysis included adenine and nicotinamide. On the basis of these facts it is suggested that the compound may consist of a dinucleotide combined with nicotinamide. Euler & Adler (65) find that this highly purified cozymase is the coenzyme of their specific alcohol dehydrogenase but is inactive for Warburg's hexosemonophosphate dehydrogenase. These two co-enzymes and their enzymic carriers are thus quite distinct though the yellow enzyme co-operates with both. Cozymase which has been heated in dilute alkali and has lost all power to activate apozymase in alcoholic fermentation still retains its activity for the formation of lactic acid from glycogen and the esterification of inorganic phosphate in muscle extracts (34). Euler & Vestin (33) conclude that cozymase has two active groups, one of which takes part in oxidation-reductions and the other in phosphorylation and lactic acid production.

Adenosinephosphates.—Lohmann has obtained adenosinediphosphate (66) by the action of washed crab muscle on adenosinetriphosphate. From the electrotitration curves of this ester, before and after hydrolysis by acids, and from other evidence (67) he concludes that adenosinetriphosphate is represented by the following formula (the diphosphate having the terminal phosphoric acid removed); the point of attachment of the ribose to the adenine residue has been determined by Gulland & Holliday from a study of the absorption spectra (private communication from the authors):



From the action of specific phosphomonoesterase and pyrophosphatase on adenosinetriphosphate, Satoh considers that the pyrophosphate group is attached directly to the third carbon atom of ribose (68); but this is not accepted by Makino (69). Lohmann & Schuster (70) found that practically the whole of the adenylic acid of

striped muscle of the frog, rabbit, and lobster exists in the form of adenosinetriphosphate. In the heart muscle of calves also, examined within a few minutes of death, adenosinetriphosphate was the major constituent but smaller amounts of adenosinediphosphate were found, the latter being possibly due to post mortem changes (71). These authors consider that the preparations of di-adenosinepentaphosphate obtained by other workers (72, 73) from heart muscle were probably mixtures of adenosine-diphosphate and -triphosphate. From Lohmann's recent work (66) it seems most probable that the reaction, adenosinetriphosphate \rightleftharpoons adenylic acid in muscle, takes place in two stages, adenosinediphosphate being an intermediate product; the removal of the first and second phosphoric acid groups is effected by different enzymes, only the second being activated by magnesium ions. Adenosinetriphosphate has also been isolated from fresh yeast (74, 75, 76), but maceration juice contains only insignificant amounts (74).

Some at least of the reactions which have been demonstrated in yeast and muscle extracts occur also in other tissues and organisms. A number of papers deal with these reactions in hemolysed red cells and in kidney and liver preparations (42, 43, 44, 77, 78, 79). Boyland & Boyland have obtained phosphodihydroxyacetone by the action of tumour extracts on hexosediphosphate (80). Neuberg and his coworkers have furnished evidence of the conversion of hexosediphosphate into a triosephosphate and of phosphoglyceric acid into pyruvic acid by preparations of various bacteria and moulds, and of germinated peas and beans (81). Tankó has obtained hexosephosphates by the action of ground peas on glucose plus phosphate (82). Burkard & Neuberg have isolated hexosemonophosphate from the leaves of the sugar beet and suggest that this may be concerned in the synthesis of sucrose (83). Evidence of an alternative path of carbohydrate breakdown by way of methylglyoxal, activated by glutathione, is given by various workers (84, 85, 86, 87) but is contested by Meyerhof (17).

VARIOUS PHOSPHORIC ESTERS

Mannosemonophosphate, previously isolated from the products of fermentation of glucose and fructose, has been obtained in high yield by fermenting mannose with dried yeast at 38° [Jephcott & Robison (88)]. Additional evidence that the phosphate group is in

position 6 is given by Patwardhan from the study of phosphomannonic acid and its lactones (89). The isolation of pure fructose-1-phosphate from the products of hydrolysis of fructosediphosphate by bone phosphatase has been completed by Tankó & Robison (90), who show that the formation of aldosephosphate in this hydrolysis is due to phosphohexokinase present in the phosphatase. Grant (91) has investigated the fermentation of galactose by preparations of adapted yeast and has confirmed the production of fructosediphosphate; trehalosemonophosphate was also isolated but no evidence was obtained of the formation of any galactose ester. The preparation of 3-phosphoglyceric acid and its salts is dealt with in papers by Neuberg and his coworkers (92, 93, 94). Phosphocreatine, apparently identical with the muscle compound, has been isolated from mammalian brain (95). Outhouse & King (146) have obtained a phosphoric ester containing amino nitrogen from malignant tissue.

The following have been synthesised: phospho-*l*-lactic acid (96); phosphocreatine, isomeric with the natural compound (97); phospho-*d*-serine (identical with the natural compound) and phospho-*l*-hydroxyproline (98); phosphoric esters of choline (99, 100), and of various aromatic alcohols (101); pyrophosphocitric acid (102).

Nucleic acids and nucleotides.—By the depolymerisation of thymus nucleic acid with extracts of intestinal mucosa in the presence of arsenate, which inhibits dephosphorylation, Klein & Thannhauser (103) have obtained the phosphodeoxyribosides of guanine, adenine, thymosine, and cytidine in pure crystalline condition. No trace of diphosphoric esters was obtained in this enzymic breakdown. In their chemical, physical, and biochemical properties these nucleotides show marked similarity to the corresponding ribosides of yeast nucleic acid. The manner in which they are linked together in thymus nucleic acid is discussed by Klein & Rossi (104) who were unable to confirm the previous findings of Takahashi (with specific phosphatases) from which he deduced a ring structure for this acid. Makino (105), from a consideration of the increased acidity following depolymerisation, assigns a ring structure to both yeast- and thymus-nucleic acids. Ribose-5-phosphate (106) and ribitol-5-phosphate (107) have been synthesised by Levene *et al.* The identity of the former with the compound obtained from muscle adenylic acid confirms Levene's previous conclusion regarding the position of the phosphate group in this nucleotide. Levene & Tipson (108) have effected the partial syntheses of two ribose nucleotides: inosine-5-

phosphate from inosine, and uridine-5-phosphate from uridine. The former was identical with muscle inosinic acid but the latter was not identical with natural uridylic acid. Levene's conclusion that the phosphoric acid group in uridylic and cytidylic acids is in position 3 receives confirmation from Brederick (109).

Phosphoric esters in marine organisms.—The occurrence of phosphoric esters has been demonstrated in marine algae (110) and in plankton (111). It is considered that organic phosphorus compounds in diatoms may pass undigested through the animals and remain in solution in the sea until regeneration to phosphate takes place in late summer.

THE MECHANISM OF CALCIFICATION IN ANIMAL TISSUES

The inorganic material of the skeleton.—The problem of the chemical nature of the bone salt is still unsolved. Marek, Wellman & Urbanyi (112) find that the composition of the inorganic portion of the bones of pigs varies with the mineral composition of the diet and deduce from this that calcium orthophosphate and calcium carbonate are precipitated separately and not as a complex salt. Klement (113) strongly contests this view, maintaining his previous opinion, based on chemical and x-ray investigations, that the bone salt consists chiefly of a hydroxyapatite. He rightly insists that the nature of the salt cannot be deduced from chemical analysis alone. Variations in the ratios of the basic and acidic elements have been frequently noted but in no way refute the evidence for the presence of complex carbonato- or hydroxy-phosphates. Brooke, Smith & Smith (114) find that the ratio $\text{Ca}_3(\text{PO}_4)_2/\text{CaCO}_3$ is greater in the bones of rats fed on a diet low in inorganic salts than in normal bones. According to Harshaw, Fritz & Titus (115) the ratio Ca/P is low in bones of newly-hatched chickens but subsequently increases. Orent, Kruse & McCollum (116) find that the bones of rats fed on diets deficient in magnesium contain unusually high proportions of ash, calcium, and phosphorus while the percentage of magnesium is very low. Deposition of magnesium does not, however, entirely cease on these diets. Useful analyses of bone, including values for elements which are rarely determined, are given by Silberstein (117). Bowes & Murray (118) give very full analytical data for the chemical composition of dentine and enamel; the inorganic portions of these tissues are not identical, that of enamel consisting largely of hydroxy-

apatite while that of dentine contains more carbonate and corresponds closely with the bone salt. The composition of bone, dentine, and enamel has also been investigated by Logan (119). From chemical analysis and x-ray diagrams Philipp finds that the inorganic portion (75 per cent) of tooth tartar consists of a hydroxyapatite containing carbonate and traces of other elements (120). The methods of analysis of bone are critically discussed by Burns & Henderson (121).

Physico-chemical relationship of blood plasma to the bone salt.—Closely linked with the nature of the bone salt is the important question of the relationship of blood plasma to this salt. McLean & Hastings have described a method for determining the concentration of calcium ions using the isolated frog's heart as indicator; values ranging from 4.5 to 5.25 mg. per 100 cc., more than twice as high as previously accepted figures, have been obtained and lead the authors to conclude that a state of supersaturation exists with respect to calcium orthophosphate and calcium carbonate. They find no evidence of the presence of a Ca-P complex in the fluids of the body (122, 123). Greenberg & Larson (124) also reject the conclusion of Benjamin & Hess, maintained by Benjamin (125), that a specific Ca-P complex of physiological importance exists in plasma. The earlier suggestion of Eichholz and Starling that colloidal $\text{Ca}_3(\text{PO}_4)_2$ may be present in serum under certain conditions is supported by various workers. These questions are fully discussed in a review by Schmidt & Greenberg (126).

*The calcifying mechanism.*⁴—The investigation of the calcification process in cartilage and bone has been continued by Robison & Rosenheim (127) who conclude that the two mechanisms which have been empirically distinguished, namely phosphatase and the "second mechanism," which induces deposition from supersaturated inorganic solutions, may form one complex enzyme system by which phosphoric esters are synthesised from inorganic phosphate and subsequently hydrolysed for the precipitation of the bone salt. Phosphatase, an essential component of this enzyme complex, is relatively stable and can effect calcification when other components have been partially inactivated (by chloroform, acetone, potassium cyanide, desiccation or in other ways) provided that pre-formed phosphoric esters are available. The conclusion that the full calcifying mechanism is enzymic in nature was partly based on the pronounced effect

⁴ Cf. also this volume, p. 303. (EDITOR.)

of iodoacetate and fluoride in extremely low concentrations, which inhibit calcification *in vitro* unless phosphoric esters are present; phosphatase is not inactivated by these agents. The calcifying mechanism is not necessarily destroyed on the death of the cells. Rosenheim (128) has shown that the calcifying power of cartilage in the bones of rats fed on a rachitogenic diet decreases as the period on the diet is increased. Such changes may be partly responsible for the deficient calcification in rickets. The gradual development of a highly specific calcifying mechanism in differentiated cartilage and osteoid tissue is further emphasized in studies of embryonic mammalian [Niven & Robison (129)] and avian tissues and tissue cultures [Fell & Robison (130)]. Cultures of osteoid and fibrous tissues were grown under similar conditions and immersed, side by side, in calcifying solutions, which produced dense deposits in the former but none in the latter. Phosphatase was synthesised by the osteoid but not by the fibrous tissue (130). Calcification of kidney, lung, and aorta has also been effected *in vitro* by prolonged immersion of the tissues in the experimental solutions (131) but the process was slow and erratic. The deposits bore some resemblance to those occurring *in vivo* in hypervitaminosis-D. That the calcifying mechanism is not entirely specific for the normal calcium salt of bone was proved by the formation of deposits of barium, strontium, and magnesium phosphates in hypertrophic cartilage, *in vitro* (131).

Kay & Skill (132) have shown that beryllium rickets, produced by addition of beryllium carbonate to the diet, is prevented by parenteral injection of sodium glycerophosphate. This adds further support to the view that the lack of calcification is mainly due to defective absorption of phosphate from the gut. From the work of Sobel, Goldfarb & Kramer (133) it would seem that the calcifying mechanism is also injured since the bones of such rats possess a markedly diminished power of calcification *in vitro*. The same authors show that severe rickets is produced by substituting strontium carbonate for calcium carbonate in the diet (134). This condition, like that of beryllium rickets, is not readily prevented or cured by administration of vitamin D. The bones exhibit a greatly diminished calcifying power when tested *in vitro*. The injury, which is reversible, is not associated with any loss of phosphatase activity but is due to the inhibitory effect of strontium ions on the calcifying mechanism. The authors conclude that strontium combines with a factor whose concentration in the bone plays an essential part in calcification.

PHOSPHATASES⁵

Schäffner & Bauer (135) have isolated from beer yeast, by extraction with glycerol, a highly specific α -glycerophosphatase, optimum pH 6.4, and claim that this enzyme provides the most exact method for the estimation of α -glycerophosphate in mixtures of α - and β -forms. They confirm these findings (136) in a reply to Schuchardt (137) who was unable to obtain preparations showing such specificity. Albers & Albers (138) found that top yeast contains one phosphatase of optimum pH 4, while bottom yeast contains in addition two others with optimum pH 7, one of these being specific for α -glycerophosphate. The hydrolysis of pure α - and β -glycerophosphates by various mammalian and plant phosphatases has been further studied by Rae, Kay & King (139) who have applied the results to the estimation of these isomerides (Kay's & Lee's method) and the purification and attempted resolution of α -glycerophosphate; no definite evidence of such resolution was obtained. This enzymic method was also used to demonstrate the presence and relative amounts of α - and β -glycerophosphates in several natural phosphatides (140). King has shown (141) that an enzyme present in intestinal mucosa and kidney liberates phosphoric acid from various phosphatides and their derivatives, bromolecithin being hydrolysed at a very much greater rate than lecithin or hydrolecithin. The acceleration of the enzymic breakdown of phosphoric esters by arsenate and arsenite has been studied by Pett & Wynne (142) who find no evidence that this is due to direct stimulation of phosphatase activity. An amylophosphatase has been isolated from barley and malt (143). The action of this and other phosphatases on starch and glycogen promises to throw light on the constitution of these polysaccharide phosphoric esters (144, 145). Further papers from Akamatsu's Institute (101) deal with the specific monoesterases and diesterases which have been used to investigate the constitution of adenosinetriphosphate and lecithin (68, 147).

Acid and alkaline phosphatases having pH optima 4.5 to 5.5 and 9.0 to 9.5 have been demonstrated in spleen (148), liver, and kidney (149, 150). The acid phosphatase of the spleen is not identical with that of the red cells (148). Separation of the two enzymes obtained from pig's liver autolysates can be effected by treatment with 0.05*N* NH_4OH or *N* CH_3COOH ; the former inactivates the acid and the latter the alkaline phosphatase (150). Differences were observed in

⁵ Cf. also this volume, pp. 43, 305. (EDITOR.)

the inactivating effect of oxalate on the alkaline phosphatases of these tissues and of bone (149). Red cells also contain both acid and alkaline phosphatases which can be separated by fractional adsorption on kaolin (151).

A detailed study of the very active phosphatase of the mammary gland has been carried out by Folley & Kay (152) who find it identical in all its properties with the alkaline phosphatase of kidney tissue: it possesses no exceptional synthetic ability. Continuing their previous work, Kay & Graham (153) have given details of two simple tests for efficiency of pasteurisation, depending on the inactivation of the milk phosphatase. The phosphatase is estimated by the convenient and highly sensitive method of King & Armstrong (154), in which the substrate is phenol phosphate and the amount of phenol set free is estimated colorimetrically by the Folin-Ciocalteu reagent. The method was devised primarily for the estimation of phosphatase activity in serum and bile; with a hydrolysis period of thirty minutes at pH 8.9 and 37.5°, the unit, expressed as milligrams of phenol per 100 cc. of serum, is almost the same, numerically, as that of Jenner & Kay.

Further evidence is given of the value of serum-phosphatase determinations in bone diseases. In rickets, the serum-phosphatase content is a better criterion of the severity of the disease and the course of healing than are the concentrations of calcium and phosphorus in the serum (155, 156). It is significantly raised in the osteoblastic type of osteogenic sarcoma and falls rapidly after surgical removal of the tumour, rising again with its recurrence. The phosphatase activity of tumour tissue of this type is also much higher than that of other types of tumour (157). A number of papers give further data on the greatly increased values of serum phosphatase in obstructive jaundice first observed by Roberts (158, 159, 160). In dogs suffering from an experimental obstruction to the common bile duct the serum phosphatase rose progressively, reaching 30 to 100 times the original values after six days (158), while large amounts of phosphatase were found in the bile. Bodansky (161), from the results of experiments with fasting dogs, concludes that only the excess phosphatase in the serum in bone diseases is derived from the bone and in hepatic jaundice from the liver; the normal level of this enzyme is determined by other factors. Very high phosphatase values were found by this author in the serum of new-born puppies and were considered to be related to active food absorption rather than to bone metabolism. In normal hens, the

serum-phosphatase activity is not greatly affected by egg-laying but in hens suffering from vitamin-D deficiency the value is increased during laying; this may be due to the withdrawal of calcium from the bones (162).

Marked increase in phosphatase activity occurs in fractured bones during healing (163, 164). Variations in the urinary phosphatase activity associated with food metabolism are shown by Kutscher & Wolbergs, who also describe the occurrence of a very active phosphatase in the human prostate gland and seminal fluid; it is not identical with the urinary phosphatase (165).

A method of estimation of the phosphatase activity of minute tissue fragments has been described and values so obtained are given for various animal tissues (166).

Many important papers in this section must be omitted; but reference may be made to the review on phosphatases by Folley & Kay in the forthcoming volume (V) of the *Ergebnisse der Enzymforschung*. Discussion of the phosphorus requirements of animal and plants and the influence of various factors on the state of the phosphorus compounds in the body must be postponed owing to lack of space.

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UNIVERSITY OF LONDON
LONDON, ENGLAND

CARBOHYDRATE METABOLISM*

By I. L. CHAIKOFF

*Division of Physiology, University of California Medical School,
Berkeley, California*

ABSORPTION OF CARBOHYDRATES¹

At the time of discovering their method for the study of the absorption of carbohydrates from the gastro-intestinal tract, Cori & Cori (1925) pointed out that the rate of absorption of sugar is, within wide limits, independent of the amount present in the intestinal tract. During the year under review this point has been investigated anew by a number of workers. From a study of a large number of animals Feyder & Pierce (50) concluded that the rate of absorption is not constant but decreases with time during a three-hour period of observation. The earlier finding, that fasting for forty-eight hours depresses the rate of absorption as compared with that noted after shorter periods of inanition, was not confirmed. No constant relation between absorption and body weight (coefficient of absorption) was found. According to Donhoffer (41), the breed of the rat plays a part in the rate of absorption of various sugars. Salter *et al.* (105) state that in mice the rate of absorption of glucose depends upon the amount administered. Cajori & Karr (16) found, in confirmation of earlier work on the rat, that, when solutions containing both glucose and galactose were introduced into Thiry loops of dog's jejunum, the total amount of carbohydrate absorbed was about the same as when each carbohydrate had been administered separately. A similar inhibition of glucose absorption does not occur in the presence of electrolytes (16, 125). Judging from the blood-sugar level in the portal vein, Meythaler & Seefisch (86) conclude that absorption of glucose introduced into the duodenum begins during the first minute and that the time of onset is independent of both the concentration of the sugar solution and the nutrition of the animal.

Further evidence that the intestinal mucosa plays more than a passive rôle in the absorption of carbohydrates has been offered by Verzár and his associates (75, 76, 83, 125). A comparison was first made of the absorption rates of isotonic solutions of glucose and

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¹ The absorption and utilization of carbohydrates have been reviewed by Pierce in *J. Nutrition*, 10, 689 (1935).

sodium sulphate (125). The selective action by which glucose is absorbed much more rapidly than the salt holds only for the intestine of the intact animal, this property being lost in the isolated gut. Laszt (75) and Laszt & Süllmann (76) have shown that phosphorylation of glucose, fructose, and galactose takes place in the intestinal mucosa during their absorption. Under the influence of monoiodoacetic acid, the phosphorylation mechanism in the intestine of the intact animal is inhibited (76), and the rate of glucose absorption is then reduced to approximately that of sodium sulphate, which latter these workers regard as due to a simple diffusion process (125). Sugars such as mannose, arabinose, and xylose, which are not rapidly absorbed, involve no phosphorylation in their passage across the intestinal wall (76).

The influence of a number of other factors on glucose absorption has been recorded. The observation that phlorhizin decreases the rate of absorption has been confirmed by Donhoffer (42). The promotion of glucose absorption by phosphates, pointed out by previous workers, has now been ascribed by Laszt (75) to a hydrogen-ion effect, for improved absorption occurs in the presence of a phosphate buffer only at a pH of 7.0. Similar effects may be produced with other buffers at the same pH. Making no reference to pH, Cajori & Karr (16) state that phosphates do not influence the rate of absorption of galactose, glucose, or fructose. The importance of stomach discharge in the study of rates of absorption has again been pointed out by McDougall (85), Donhoffer (41), and Kerly & Reid (71).

GLUCOSE TOLERANCE AND BLOOD SUGAR

Three interpretations of the glucose-tolerance curve have been presented: (a) Himsworth (63) deduced that the glucose tolerance, as influenced by the diet, is an expression of the subject's sensitivity to the insulin secreted by his own pancreas, and that this secretion is constant in amount irrespective of the tolerance shown by the curve. He states also that the sensitivity depends solely on the carbohydrate in the diet. (b) Soskin *et al.* (117, 118) restate their belief that the liver, and not the islet tissue, limits the tolerance for glucose observed in toxic conditions and in hunger diabetes. (c) Clark, Gibson & Paul (28), however, conclude that the normal glucose-tolerance curve is related to the amount of endogenous insulin secreted by the subject's own pancreas, which can be suppressed by the administration of exogenous insulin. They injected large doses of insulin, up to

75 units daily, for several weeks in normal subjects and made the interesting observation that, when the insulin was discontinued, a temporary decrease in glucose tolerance set in, which was associated with an occasional glycosuria. There can be no doubt that the liver is concerned in determining the degree of glucose tolerance; this fact has long been recognized. On the other hand, the earlier observations of Murray & Walters (1932) indicate that in an acute infection, a condition in which the glucose tolerance is known to be decreased, the insulin content of the pancreas may be altered. The glucose-tolerance curve is probably the result of a number of factors, and, in the absence of more specific indicators of the insulin content of the blood, alterations in the insulin secretion by the pancreas can hardly be dismissed on the mere evidence of blood-sugar changes.

Somogyi (1933) observed that the corpuscles in some cases are sugar-free. Olmsted (93) now reports that, when proper precautions are taken, the corpuscles of the rabbit, rat, guinea pig, cat, dog, and man can be shown to contain no glucose, regardless of the level of the blood sugar. The presence of glucose in the red blood corpuscles obtained from oxalated blood is ascribed to a change in the permeability of the cell.

In moderate activity, in which no lactic acid rise occurred, Dill, Edwards & Mead (38) found that the blood sugar remained close to the resting level. In more severe exercise, in which the blood lactic acid rose and exhaustion took place in ten to forty minutes, the blood sugar showed a significant rise during both the exercise and recovery. In short bursts of maximal exertion, which brought on exhaustion in three minutes, the blood-sugar changes were not marked. Schlutz, Hastings & Morse (107) found that the serum sugar of dogs undergoing exercise may be normal or even increased during the first fifteen minutes, whereas with more prolonged exercise a fall in blood sugar was observed in some cases. According to Chambers, Himwich & Kennard (25) the sugar excretion of fasted depancreatized dogs increases early in recovery from exercise but later decreases.

The relation of circulatory disturbances to the blood-sugar level has been studied by Kosterlitz (73) and Robertson (101). The former demonstrated that in rats in which the glycogen reserves are greatly reduced a fall in blood pressure below a critical level results in a fall in blood sugar. In confirmation of previous work, Robertson (101) found that immediately after hemorrhage there is a rise in blood sugar, the degree of which is related to the amount of blood lost.

Striking variations in the rates of glycolysis of the blood of different animals have again been reported by Hsu (67), Cutler (34), and Morgulis & Munsell (90). Hsu attributes an important rôle in this process to leucocytes. The relation of the phosphorus compounds in blood glycolysis has been studied by Morgulis & Munsell (90), Cutler (34), and Dische (39). Morgulis & Munsell found no relation between the glycolytic activity and the variations in a number of phosphorus fractions of the blood. In the goat, an animal in which the normal blood sugar is unusually low, Cutler showed that glycolysis was not accompanied by changes in inorganic phosphorus such as those found in the dog, rabbit, and man. In defibrinated or citrated blood the loss of glucose during short intervals can be accounted for by the appearance of lactic acid (34, 40).

The data obtained by Heim, Thomson & Bartter (60) and by Clark & Winter (29) show that the glucose content of lymph is very close to that of the blood plasma. When glucose was administered either intravenously or enterally, the rise and decline in the sugar curve for lymph paralleled closely that for plasma; this indicates that glucose not only gains entrance into the lymph stream rapidly, but also is rapidly removed (60). Fay & Wharton (48) found that galactose appears in the lymph during its absorption from the intestine. Gregeresen & Wright (58) report that the glucose in cerebrospinal fluid, which in the fasted dog is between 65 and 93 mg. per cent, was increased by the intravenous administration of glucose.

GLYCOGEN

Feyder & Pierce (50) compared the rates of glycogen formation from sucrose and glucose during their absorption from the gastrointestinal tract. No significant difference was found for the first two hours. During the third hour the rate of glycogenesis from sucrose exceeded that from glucose by 44 per cent. The superiority of sucrose as a former of glycogen is ascribed to the presence of fructose in the sugar molecule. Curiously enough, sucrose has a greater fattening effect on rats than an isodynamic amount of glucose (49). Although Feyder & Pierce found that the rates of absorption of different carbohydrates do not parallel their rates of glycogenesis in the liver, Salter, Robb & Scharles (105) point out that, as regards glucose, the maximum amount of glycogen stored in the liver is roughly proportional to the glucose fed. Abelin (1) found that, for a given

amount of sucrose, rats previously placed on a fat diet produced 40 to 60 per cent more liver- and muscle-glycogen than animals on a mixed diet.

The behavior of liver- and muscle-glycogen under the influence of exercise has been studied in the rat by Brand & Krogh (12). After a period of exercise, in which the stores of carbohydrate were reduced, a slight endogenous formation of glycogen from non-carbohydrate sources occurred in both liver and muscle. An increase in liver glycogen, unaccompanied, however, by a change in muscle glycogen, was also observed by Láncoz (74) in the period of recuperation following the exposure of fasted mice to cold for four to five hours. Although in neither case was it possible to decide whether the new carbohydrate had been derived from fat or protein, Brand & Krogh are inclined to regard protein as the source in their experiments.

Glycogen storage in the gastrocnemius muscle of the rat is not under the control of the nervous system. This was shown by Hines & Knowlton (64), who found that the glycogen content of this muscle is not significantly changed forty-eight hours after sectioning the sciatic nerve. Moreover, normal glycogen storage occurred when glucose was administered to well-fed animals twenty-four hours after the operation.

Stuart & Higgins (121) found that during the fluctuations produced by fasting and the ingestion of food fetal liver contained more glycogen than did maternal liver. Corey (31) also observed a rise in fetal liver glycogen after the intraperitoneal administration of glucose to the mother. He has shown that adrenalectomy and the injection of insulin in the mother reduce the liver glycogen in the fetus as well as in the mother. Placental glycogen is apparently uninfluenced by alterations in the carbohydrate metabolism of the maternal organism.

The ease with which glycogen in muscle breaks down, particularly when any interference with this tissue is attempted, has always made difficult the accurate estimation of muscle glycogen in the intact animal. The methods for the determination of tissue carbohydrates have been reinvestigated by Blatherwick *et al.* (8), employing in large measure the procedures previously reported by other workers. Muscles that had been frozen *in situ* contained more glycogen and less lactic acid than muscle frozen after the hind limb had been severed from the body. This, however, was not the case with liver, for rapid removal and immediate freezing of this tissue resulted in values for glycogen and lactic acid comparable with those obtained by freezing

in situ. According to Steiner (120), however, the freezing of muscle cannot be relied upon to yield absolute values for glycogen. He observed that the contraction elicited by freezing results in a small loss of glycogen, a loss that is reflected in a rise in both fermentable sugar and hexosemonophosphate. The procedure adopted by Steiner for the determination of sugar in muscle consisted in immersing the tissue immediately after excision in 1 *N* H₂SO₄ cooled to -4° . In studies involving comparison of glycogen contents of tissues, due regard should also be paid to sex, for, in confirmation of previous work, Blatherwick *et al.* (8) found that more glycogen was present in liver and muscle in male rats than in females.

Bott & Wilson (11) stress the importance of the intact cell structure in the production of lactic acid in the mammalian liver after its removal from the body and ascribe the failure of earlier workers to demonstrate lactic acid formation by excised liver tissue to the destruction of the mechanism responsible for its production. In muscle, however, lactic acid appeared at about the same rate, irrespective of whether it was hashed or sliced. Sharp (114) found that post-mortem glycogenolysis proceeds in fish liver even at 0° , lactic acid as well as free sugar accumulating during this process. In frozen fish muscle he showed that as the temperature falls below 0° the rate of glycogenolysis first increases (reaching a maximum between -3.2° and -3.7°) and then decreases.

Barbour's observation (1929) that a trisaccharide is the sole product of the hydrolysis of glycogen by glycerol extracts of muscle has not been confirmed by Carruthers & Lee (18). Failure to isolate a trisaccharide from the hydrolysate of glycogen has also been reported by Gray (56). According to Carruthers (17, 18), a mixture of sugars, notably maltose, is produced by this hydrolysis. Moreover, under suitable conditions a complete conversion of glycogen to glucose was found with extracts obtained from both liver and muscle. Evidence for an intermediate substance during the breakdown of glycogen in liver has also been presented by Noltie (92), Sharp (114), and Purves (98).

GALACTOSE, LACTOSE, AND FRUCTOSE

The fate of galactose in the animal body has been actively studied during 1935. Roe, Gilman & Cowgill (103) suggested that galactose as such is not oxidized by the dog since they found that the ingestion of 2 gm. of galactose per kilo of body weight did not lead to a sig-

nificant rise in the respiratory quotient despite the fact that the galactose content of the blood rose higher than 200 mg. per cent. When larger amounts, namely 5 gm. or more per kilo, were administered, a delayed rise in the respiratory quotient occurred, which was attributed to the extra glucose derived from the conversion of galactose in the liver. Further evidence that galactose is not utilized directly by the tissues was provided by a study of the galactose content of arterial and venous blood derived from muscle, brain, and liver while galactose was being supplied to these three tissues (102). A constant and significant removal of galactose from blood appeared in the case of only one of these tissues, namely the liver. Wierzuchowski & Fisz (126) also found that during its injection more galactose is removed by the liver per gram of tissue than by any other organ. There is a difference between man and the dog in the response of the respiratory quotient to galactose, for, in confirmation of Schmiedt (108) and of earlier workers, Roe *et al.* (103) found that a significant rise in the quotient followed within one hour the administration of 40 gm. of galactose to the human subject.

A careful study of the rôle of the liver in the utilization of galactose by the dog was made by Bollman, Mann & Power (10). Galactose, introduced intravenously in doses of 500 mg. per kilo, disappeared from the blood of normal dogs in about two hours, 10 to 30 per cent of it appearing in the urine. Galactose also disappeared from the blood of the completely hepatectomized dog, but a larger part, namely 50 to 60 per cent, of the amount injected was excreted. Since the galactose not excreted did not influence the fall in the blood sugar that occurs in the liverless dog, it seems unlikely that the retained galactose was converted to glucose. It is worthy of note that dogs in which extensive cirrhosis of the liver had been produced and those in which 50 to 70 per cent of the hepatic tissue had been excised failed to show an increased elimination of galactose. When, however, acute degenerative changes were induced in the liver by such agents as carbon tetrachloride, chloroform, etc., increased amounts of galactose were found in the urine. Reports on the metabolism of administered galactose in patients suffering from various forms and degrees of liver damage have been made by Bensley (6), Schmiedt (108) and Althausen & Wever (2).

The question of the effect of galactose upon blood glucose continues to yield conflicting results. Fay & Wharton (48) draw no conclusions on this point. According to Roe & Cowgill (102) the

nutritional state determines whether galactose raises the blood glucose in rabbits. The intraperitoneal injection of galactose into non-fasted rabbits led to a rise in the blood glucose, whereas no significant change occurred in fasted rabbits that had received similar treatment. Grant (55), however, points out that the positive results obtained in the well-fed animals may be explained by hepatic glycogenolysis. MacKay, Bergman & Barnes (80) found no difference in the sugar curves resulting from intravenously administered galactose in fasted and fed rabbits. No significant change in blood-glucose level was observed by Wierzuchowski & Fiszal (126) during the injection of galactose.

The lactose content of the blood and urine of pregnant and lactating women has been studied by Hubbard & Brock (68), who developed a method for measuring lactose in concentrations as low as 1 to 2 mg. per 100 cc. Lactose could not be detected in the plasma of either normal or pregnant women, but small amounts of it, about 2 mg. per cent, were not infrequently found in the blood of lactating women between the third and eighth day after delivery. Since lactose may be present in urine during lactation (14), although absent in the blood, they concluded that the renal threshold for lactose is very low.

Early studies of the perfusion of the excised mammary gland of sheep showed that glucose served as a precursor of lactose. No synthesis of the disaccharide occurred when galactose was added to the perfusion medium. These observations have been confirmed by Grant (55) in a study of the synthetic activity of tissue slices obtained from the mammary gland of lactating guinea pigs. Lactose was synthesized from glucose, but, strangely enough, there was little evidence for such a conversion from galactose, fructose, and mannose. Tolstoi (123) has shown that in diabetic patients in whom wide fluctuations of the blood sugar were produced by glucose and insulin practically no variation occurred in the concentration of the lactose in breast milk.

The response of the diabetic organism to lactose and galactose differs from normal. Although the administration of 1.5 gm. of lactose per kilo failed to increase the blood sugar of normal human subjects, a marked rise followed the ingestion of this amount in the diabetic patient [Koehler *et al.* (72)]. Roe *et al.* (103) observed that galactose, in doses producing significant increases in the respiratory quotient of normal dogs, was without effect on the quotient after pancreatectomy.

A relation of lactose and galactose to cataract formation has been demonstrated in the rat. Mitchell & Dodge (88) found that a diet

containing a high percentage of lactose produced mature bilateral cataracts, and this has been confirmed by Yudkin & Arnold (128) and by Day (35). Rats receiving glucose, starch, maltose, dextrin, or sucrose in place of the lactose failed to show lenticular opacities. Galactose, however, when fed at high levels by Mitchell (87) and by Yudkin & Arnold (128), produced lenticular changes. The formation of cataracts by lactose is apparently peculiar to the rat, for they do not occur in rabbits or kittens fed a high lactose diet (88).

Methods for the determination of fructose in blood have been reported by Patterson (95) and Scott (111). Wierzuchowski *et al.* (127) found that during the infusion of fructose the liver, instead of removing lactic acid from the circulation (as it does under normal fasting conditions or during the administration of glucose or galactose), contributes large amounts of lactic acid to the blood stream.

INSULIN, EPINEPHRINE, AND ADRENAL CORTEX

It was pointed out several years ago by Cori & Cori that in addition to the mobilization of liver glycogen two other mechanisms are responsible for the sustained hyperglycemia that can be induced by epinephrine: a new formation of liver glycogen at the expense of muscle glycogen, and an impaired utilization of blood sugar in the peripheral tissues. Recent work has dealt with both of these mechanisms and will be considered in the above order.

a) From a comparison of the effects of epinephrine on normal dogs, and depancreatized dogs in which the blood sugar had been maintained at normal levels with insulin, Chidsey & Dye (27) were led to conclude that the synthesis of glycogen in the liver, observed as a delayed effect of the subcutaneous administration of epinephrine, is related to an increased secretion of insulin induced by the epinephrine. A similar view has been offered by Bridge & Noltie (13), who failed to find a synthesis of liver glycogen during the continuous intravenous injection of epinephrine in unanesthetized rabbits.

b) The early belief that the utilization of blood sugar is impaired under the influence of epinephrine was based in part on studies of the respiratory quotient and arteriovenous blood-sugar differences. The results obtained in recent investigations with the same methods have been so conflicting that no conclusion may be drawn at the present time about the effect of this hormone on carbohydrate utilization. Bridge & Noltie (13) found a fall in the respiratory quotient during

the continuous infusion of epinephrine in unanesthetized rabbits. This is regarded as particularly significant in view of the fact that it occurred in the presence of a high blood sugar, a condition that in the normal animal acts as a stimulus to carbohydrate oxidation and a rise in the respiratory quotient. Dill, Edwards & De Meio (37), however, judging from the respiratory quotient, concluded that the administration of epinephrine facilitates the oxidation of carbohydrate in subjects undergoing moderate exercise. In a careful study Cori, Fisher & Cori (32) reinvestigated the significance of the arteriovenous blood-sugar differences, observed under the influence of epinephrine, as an index of carbohydrate utilization. They stress the importance of protecting dilute epinephrine solutions from destruction during prolonged intravenous administration and express the view that in the experiments of Soskin² *et al.* (from which the latter concluded that the utilization of carbohydrate is not suppressed by epinephrine) inactivation of the hormone occurred before it reached the animal. Although Cori *et al.* regard their evidence on arteriovenous blood-sugar differences as still in line with the view that epinephrine depresses carbohydrate utilization, they nevertheless point out that at best arteriovenous differences in blood sugar are a qualitative rather than a quantitative index of sugar utilization by the tissues.

In narcotized dogs Bürger & Kohl (15) and Vendég (124) found that the hypoglycemia produced by the intravenous injection of single or repeated doses of insulin favors a fall in liver glycogen. Reid (100), however, points out that during the continuous intravenous infusion of insulin in fasted cats glycogen synthesis occurs even in the presence of hypoglycemia (without convulsions). Cessation of the hormone administration resulted in a rapid glycogenolysis.

It now seems well established that the effects produced on carbohydrate metabolism by the injection of insulin or epinephrine into the normal animal are not due solely to the action of whichever hormone is administered but also involves an endogenous secretion of the other. Such an interplay of these two hormones was discovered in purine metabolism. Chaikoff & Larson (23) found that the injection of insulin in the Dalmatian coach dog led to a rise in blood uric acid. Since the rise did not occur when sufficient glucose was administered to prevent the fall in blood sugar, it was suggested that the increase in blood uric acid was secondary to the release of epinephrine, an infer-

² Cf. Cori, C. F., and Cori, G. T., *Ann. Rev. Biochem.*, 4, 195 (1935)

ence supported by the finding that epinephrine led to a marked and immediate rise in the concentration of uric acid in the blood (24).

Since 1923, cases of hyperinsulinism have repeatedly been found in man. An observation of this condition has been made by Slye & Wells (116) in a dog that suffered from attacks of hypoglycemia, which were relieved by the administration of sugar. Necropsy revealed nodules with the histological characteristics of islet tissue in the pancreas, as well as an adjacent lymph node invaded by the tumor tissue. A case of spontaneous hypoglycemia (43 mg. per cent) in an infant removed by cesarean section from a diabetic mother has been reported by Randall & Rynearson (99). Two hours before the hypoglycemia developed, the blood sugars in umbilical cord and mother were 194 and 280 mg. per cent, respectively.

The rôle of the metals presents an interesting development in the chemical and physiological action of insulin. Scott & Fisher (110) found that the ash content of crystalline insulin obtained by means of zinc, cobalt, or cadmium was directly proportional to the atomic weight of the metal used to facilitate crystallization. They, therefore, believe that the metals were in chemical combination with the insulin. No relation, however, was found between the zinc content and the amount of insulin extracted from fresh pancreas. When zinc salts were added to insulin solutions prior to their administration, a delay in the physiological response was observed. Although the blood sugar did not fall so low in rabbits that had received zinc-treated insulin as in those injected with equal amounts of untreated insulin, nevertheless the blood sugar was maintained below normal for a much longer period in the former than in the latter. Maxwell & Bischoff (84) found that the hypoglycemic effect could be not only prolonged but also intensified by the addition of dilute aqueous solutions of ferric chloride to insulin. This is ascribed to a retardation in the rate of insulin absorption in the tissues. A method for delaying the uptake of insulin from the subcutaneous tissues would, of course, prove of value in the treatment of diabetes. It is interesting to record here that the absorption of insulin from routes other than the subcutaneous has been claimed by Hermann & Kassowitz (62) and by Pribram (97), from the surface of the skin, and by Major (82), from its introduction into the nasal passages.

The loss of carbohydrate from the blood, liver, and muscle after adrenalectomy has again been investigated by Thaddea (122) and by Silvette & Britton (115). The latter have re-emphasized the impor-

tance of carbohydrate depletion as a cause of death following removal of both glands. Kendall (70) cites the work of Allers, who kept adrenalectomized dogs alive for eighty-four and one hundred and fifteen days by means of a balanced diet containing sodium chloride, sodium citrate, and a small amount of potassium. Since these animals were maintained in excellent condition and with normal blood sugars, Kendall believes that the adrenal cortex extract is not directly concerned with carbohydrate metabolism. Although Thaddea (122) states that intraperitoneal injections of the cortical hormone raise the blood sugar of normal animals, no such effect with oral administration of overdoses of the hormone was found by Grollman & Firor (59), who attribute the blood-sugar changes observed by other workers to impurities. Thaddea (122) found that the response of the adrenalectomized animal to epinephrine (as judged by the increases in lactic acid and blood sugar) is less than in the normal. He also noted that the impaired glycogen formation from glucose and lactic acid observed after removal of these glands is corrected by the administration of cortical extract.

PITUITARY³

Greely (57) observed that the blood sugar in rabbits begins to fall eleven to thirty-two hours after hypophysectomy. In this connection it is interesting to note that Cope & Marks (1934) found that increased sensitivity usually sets in within forty-eight hours after the operation. In order to maintain a blood sugar in the neighborhood of 80 mg. per cent in fasted hypophysectomized rabbits Greely determined that between 0.5 and 0.7 gm. of glucose is required per kilo per hour, an amount that, according to Drury (43), is far in excess of that necessary to keep the blood sugar in eviscerated rabbits at normal levels. According to Chambers, Sweet & Chandler (26), the low blood sugar in the hypophysectomized dog is not due to an increased ability to oxidize carbohydrate.

The relation of cerebral manipulation as well as of hypophysectomy to the response of the blood sugar and inorganic phosphorus to insulin was investigated by Chaikoff, Reichert, Larson & Mathes (21). Since hypophysectomy by the intracranial route involves considerable cerebral manipulation before the gland can be seen and removed, control animals were provided in which all cerebral manipulations were

³ Cf. also this volume, p. 329. (EDITOR.)

carried out up to, but not including, excision of the gland. The occurrence of a low blood sugar in the postabsorptive state is not a constant finding in completely hypophysectomized dogs. Curiously enough, a variable but none the less significant degree of increased insulin sensitivity was observed in the operated controls. Although histological examination of the pituitary gland at necropsy showed the glands to be normal, there can be little doubt that the sensitivity in this case was the result of some interference in the hypophyseal region. Inasmuch as the technical details employed in the removal of the gland vary widely among different operators, it does not necessarily follow that the sensitivity observed by Chaikoff, Reichert *et al.* in their operated controls plays a part in the hypophysectomies performed by other workers. It should be noted here that Keller, Noble & Keller (69) reported that hypoglycemia in dogs may be produced by hypothalamic lesions in which no pituitary damage is involved.

From the changes produced in three blood constituents, namely the rise in glucose and lactic acid and the fall in inorganic phosphorus, Chaikoff, Reichert, Read & Mathes (22) concluded that the completely hypophysectomized dog is capable of responding to epinephrine, though subnormally. Since no interference in the capacity to store glycogen was found in dogs deprived of all hypophyseal tissue for as long as four months (20), it was suggested, as previously pointed out by Corkill, Marks & White (1934), that in the absence of hypophyseal hormones there is an interference in the mechanism whereby glycogen from muscle and liver is made available for blood-sugar purposes. This conclusion is in keeping with the observation of Fluch, Greiner & Loewi (52) that excision of the anterior lobe decreases the rate of hepatic glycogenolysis in the frog.

Houssay's observation that hypophysectomy prolongs the life of the depancreatized animal not receiving insulin has been confirmed by Mahoney (81) in the monkey and by Soskin *et al.* (119) in the dog. Despite this, however, such animals are not free from the signs and symptoms of diabetes. In the writer's laboratory a hypophysectomized-depancreatized dog was maintained without insulin from February 19 to June 4, 1935, at which time it was sacrificed for glycogen and fat determinations (unpublished observation). During this interval the dog dropped in weight from 10.2 to 5.8 kilos. The blood sugars varied from 170 to 292 mg. per cent. With the exception of two days glucose was always present in the urine. On June 4 the liver contained 2.7 per cent glycogen and 38 per cent fatty acids. The view

of Shapiro (113) that glycogen formation is necessary for the prevention of ketosis is borne out by the fact that in the hypophysectomized dog a lowered ketone-body excretion is associated with a marked retention of the capacity to store glycogen in the liver.

Selle, Westra & Johnson (112) are not in agreement with the claim of Barnes, Culpepper & Hutton (4) that the irradiation of the hypophysis of depancreatized dogs decreases the severity of diabetes. Since attempts have already been made to treat clinical cases of diabetes by irradiation of the pituitary region, a word of caution is in order: in view of the many functions of the hypophysis, the possibility of deleterious effects of such treatment—even if claims for its beneficial effect on diabetes should be substantiated—requires careful consideration.

Because of the intimate relation between adrenal and pituitary glands, investigations of the influence of adrenalectomy upon the intensity of pancreatic diabetes may be considered at this point.⁴ Long & Lukens (79) compared the effects of adrenalectomy and hypophysectomy upon depancreatized cats. Those depancreatized survived for two to five days, those adrenalectomized-depancreatized for eight to twenty-eight days, and those hypophysectomized-depancreatized for eighteen to eighty-five days. In the two latter cases the excretion of ketone bodies and glucose was less than in depancreatized animals. According to Thaddea (122), in depancreatized cats the blood sugar as well as the liver- and muscle-glycogen are lowered by excision of the adrenals. Ferrill, Rogoff & Barnes (51) found that a reduction of the insulin requirement of depancreatized dogs followed adrenalectomy. On the other hand, Gondard, Hédon & Loubatières (54) state that the intensity of diabetes, as judged by D:N ratios, is not appreciably lessened in animals that have survived adrenalectomy for brief periods.

During the past few years there have appeared numerous papers in which it is maintained that hypophyseal extracts can counteract the insulin sensitivity that follows excision of the pituitary gland, besides producing glycosuria and hyperglycemia in normal animals. Collip (30) has summarized the earlier work in this field. The papers to be discussed here are grouped according to whether or not a change in carbohydrate metabolism was produced by administration of the extracts employed.

Negative results.—Hrubetz (66) injected growth hormone, in

⁴ See also section on adrenal cortex.

doses she regarded as within the physiological range, into rats for several days but failed to find a rise in the blood sugar. She also points out that the dose employed by Houssay *et al.*, who obtained changes in the blood sugar in rats injected with pituitary extracts, was sufficient to distend the animal's abdomen. Nelson, Turner & Overholser (91) used a lactogenic hormone that they believed to be free from other pituitary factors. Its injection into four monkeys produced neither hyperglycemia nor glycosuria. Anselmino & Hoffmann (3) were unable to confirm Lucke in his claim to have found a rise in the blood sugar within one hour after a single injection of praephyson (a commercial preparation of the anterior lobe).

Positive results.—Anselmino & Hoffmann (3) studied the effects of aqueous extracts of acetone-dried powders of beef anterior lobe on carbohydrate metabolism. Confirming earlier observations, they found a delayed response in the blood sugar of dogs subjected for fourteen days to daily injections of an extract corresponding to 250 mg. of the dried powder per kilo of body weight. They further claim that the anterior lobe contains a "pancreatropic" hormone which, by stimulating the islets, lowers the blood sugar. The administration of fractions in which the sugar-lowering factor had been destroyed results in an immediate rise in blood sugar, the maximum being observed five hours after the injection. A lowering in the blood sugar together with an increased secretion of insulin following the administration of the "pancreatropic" hormone is also claimed by Zunz & La Barre (129).

Rothschild & Staub (104) observed hyperglycemia in rabbits after single and repeated injections of thyreotropic hormone. Zunz & La Barre, however, state that commercial preparations of the same hormone lower the blood sugar (130).

The belief held by some that the anterior lobe contains a diabetogenic hormone rests on the increased sensitivity to insulin that follows hypophysectomy, the decrease in the diabetic signs in depancreatized animals in which the hypophysis has been excised, and the preparation of extracts that produce glycosuria and hyperglycemia. Until the rôle of the thyroid and adrenal glands in the metabolism of hypophysectomized and hypophysectomized-depancreatized animals is understood, it seems premature to invoke a diabetogenic hormone as an explanation of the carbohydrate changes in such animals. Moreover, the significance of extracts that produce hyperglycemia in normal animals as well as nullify the insulin sensitivity of those hypophysectomized

is far from clear. The lack of uniformity in the response of the blood sugar to the extracts employed by different workers is indeed striking. No significance can be attached to a hyperglycemia or to a glycosuria that appears after several daily injections of massive amounts of a crude extract, or to one that appears after several months of daily treatment with such fractions. It is imperative to recognize that not every rise or fall in blood sugar is evidence for a new carbohydrate-metabolism hormone. It is by no means unlikely that with greater purification of the extracts in use much of the effect on blood sugar will disappear.

The influence of the vasopressor and oxytocic fractions of the posterior pituitary gland upon blood sugar has been studied by Zunz & La Barre (131) and by Holman & Ellsworth (45, 65). The latter found that, although ineffective in the rabbit (45), in the dog both fractions were active in raising the blood sugar when administered in small doses (65). Since the oxytocic was twenty times as effective as the vasopressor principle, the hyperglycemic action of the latter was ascribed to its known contamination with oxytocin. It is also pointed out that the hyperglycemic effect of large doses of vasopressin may be secondary to a circulatory disturbance.

DIABETES⁵

The early attempts by Macleod to maintain the completely depancreatized dog with insulin for periods longer than eight months met with no success until raw pancreas was included in the diet. More recently Best and his associates have claimed that choline is the accessory substance required for the maintenance of the depancreatized dog receiving insulin. Chaikoff (19), however, has now kept two depancreatized dogs alive for well over four years by means of insulin and a diet containing the nutritional requirements known to be essential for the normal dog, but without the addition of supplementary amounts of pancreas, lecithin, or choline. Despite their survival under these conditions, the two animals are not normal in all respects, the most significant change from the metabolic point of view being the development of livers characterized by marked infiltration of fat. In carbohydrate studies involving the depancreatized dog, due regard therefore should be paid to the state of the liver, for otherwise there

⁵ See also section on pituitary.

is no way of distinguishing between the effects produced by the absence of the pancreas and those produced by an abnormal liver.

Evidence for the presence of a duodenal factor that can influence the level of the blood sugar has again been presented by Laughton & Macallum (77) and Heller (61). Beneficial effects from the use of the Macallum-Laughton extract in the treatment of diabetes are claimed by Duncan *et al.* (44).

In rats rendered resistant to the ketogenic factor by prolonged administration of anterior pituitary extracts, Black (7) found that, although the ketone-body excretion produced by phlorhizin was far less than in a normal rat receiving this drug, the sugar output was not significantly different in the two types of animals. From a study of blood samples taken from various vessels, London *et al.* (78) concluded that the effects of phlorhizin involve organs in addition to the kidneys and a toxic disturbance more widespread than a glycosuria.

THE CONVERSION OF FATTY ACIDS TO CARBOHYDRATE

Although this controversial question still holds the attention of a number of workers, no evidence has yet been offered proving conclusively that the conversion can or cannot occur in the mammalian organism. A synthesis of carbohydrate during the incubation of isolated liver slices was observed by Gemmill & Holmes (53) and by Barreda (5). Because this was accompanied by a respiratory quotient below 0.7 in liver slices obtained from rats fed a high fat diet, Gemmill & Holmes claim that the new formation of carbohydrate had taken place at the expense of fat. This conclusion, however, has been criticized by Cori & Shine (33), who point out that the addition of α - or β -glycerophosphate or of glycerol to liver slices taken from fasted rats also results in an increase in fermentable carbohydrate. Mirsky & Soskin (89) believe that the increase in the total carbohydrate of rats under the influence of dinitrophenol is derived from fatty acids, since the new formation of carbohydrate is associated with a decrease in the lipid but not in the nitrogen content of the animal. But this was not a consistent finding, for under similar treatment rats also showed phases in which the carbohydrate decreased and the fat remained unchanged. Soskin *et al.* (119) believe that the hypophysectomized dog differs from the normal in being unable to convert fat to carbohydrate—a view that can be held only when this conversion is accepted for the normal animal.

The early attempts to demonstrate a glycogen formation from

ingested fatty acids met with little success. This problem has been reinvestigated by Deuel *et al.* (36). No evidence was found for the formation of glycogen from even-chained fatty acids.⁶ Increased glycogen deposition did occur, however, after the feeding of odd-chained fatty acids. In view of the recent work of Schoenheimer & Rittenberg (109), in which, by the aid of deuterium, it was shown that the largest part of fat in the diet is deposited in the depots prior to utilization, it would seem that the significance of glycogen changes in the liver after fat feeding would be enhanced if the fate of the ingested fatty acid were known.

HEART

Since glycolysis by the lungs could not be adequately controlled in the heart-lung preparation, Evans *et al.* (46, 47) employed the isolated heart-oxygenator circuit to redetermine the glucose and lactic acid utilization of cardiac tissue. They reported that a dog's heart under apparently normal conditions of work, pressure, and temperature uses on the average 70 mg. of glucose and 200 mg. of lactic acid per 100 gm. of tissue per hour. The diabetic heart removed 35 mg. of glucose as compared with a value of 150 mg. utilized by the normal heart in which the circulating blood glucose had been raised to diabetic levels. The consumption of glucose by both diabetic and normal hearts was speeded up by the addition of insulin. The lactate utilized by the diabetic heart was not significantly abnormal. A lowered utilization of mannose, fructose, and galactose as well as of glucose by the diabetic heart has been found by Pomóthy (96). Pal & Prasad (94) report that the presence of lactose, maltose, fructose, or sucrose in the perfusion medium improves the contraction of a frog's heart and that the effect of these carbohydrates is further increased by the addition of insulin.

Scheinfinkel's observation (106) that glucose utilization by a frog's heart is the same regardless of the work performed was not confirmed in the dog by Bogue *et al.* (9). They showed that a rise in the energy output induced either by epinephrine or by increasing the mechanical work performed was accompanied by an increase in the utilization of both lactic acid and glucose.

It is regretted that the space allotted does not permit reference to

⁶ In surviving tissues, however, Haarmann [*Biochem. Z.*, 282, 406 (1935)] has reported the formation of lactic acid from butyric and β -oxybutyric acids.

a number of interesting papers published during the year under review. The assistance of Mr. A. Kaplan, Mr. G. E. Gibbs, and Dr. A. E. Gordon in the preparation of this manuscript is gratefully acknowledged.

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DIVISION OF PHYSIOLOGY
UNIVERSITY OF CALIFORNIA MEDICAL SCHOOL
BERKELEY, CALIFORNIA

FAT METABOLISM*

BY EMILE F. TERROINE

*L'Institut de Physiologie Générale de la Faculté des Sciences, l'Université de
Strasbourg, Strasbourg, France*

FATTY COMPONENTS OF THE ORGANISM

The distinction made by Terroine (1919) between two categories of lipids, essentially different both chemically and physiologically, the *élément variable* consisting of the reserve fat and the *élément constant* representing the essential component of the protoplasm, has generally been adopted. The characteristics and properties of these two elements have been more exactly determined during the year 1935, but some of the qualities formerly attributed to them have since been disputed.

ÉLÉMENT CONSTANT

Terroine & Belin, who relate the *élément constant* to the phospholipins have found this fraction quantitatively and qualitatively constant in spite of all physiological variations. It is characteristic in the tissues for a given cell species. Different investigations serve to establish these conclusions.

Quantitative constancy and total amount.—Alf Klem has studied the quantitative constancy in the whole organism; two rats, starved to death, gave 1.58 and 1.54 per cent of fatty acids, values which are identical. Valla has made investigations upon a great number of mice and has shown that the value found is not absolutely the same, but deviates strikingly little from the mean; the value of the *élément constant*, which has recently been better estimated than by Terroine, is for mice 2.0 per cent of the body weight. The important variations which a different external temperature induces in the survival do not modify the final value, a phenomenon seen formerly by Terroine & Barthélémy. As to the biometric point of view, it would be very interesting to determine the *élément constant* by the proportion of fatty acids in the whole organism at the moment of death to total nitrogen; in this way the value would be more exactly grouped than when related to total weight, which is always an equivocal factor. The latter observation can be applied to the substances which always appear with the phospholipins: cholesterol and unsaponifiable material.

The notion of a quantitative constancy in a lipid element essen-

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tially represented by phospholipins, is not in complete agreement with the results of investigations made on individual tissues and on isolated cells.

Boyd sees in the variations of the phospholipins (as well as in the associated variations in free and esterified sterols) an index of the physiological activity of the whole organism or of a certain tissue. His chief arguments are: after an operation, of any cause whatever, the phospholipins of the white corpuscles increase by 200 per cent if recovery sets in and fall by a like proportion if post-operative complications ensue [Boyd (1)]; after fever, if recovery is normal, the leukocytes increase in phospholipin content, but suffer a decrease if the illness proceeds fatally; just after parturition in the rabbit the phospholipins of the white corpuscles increase and return to normal after three weeks [Boyd (2)]; the phospholipin content of the rabbit ovary increases at the beginning of pregnancy and in fourteen or sixteen days reaches 300 per cent of its normal value, returning to normal just before parturition [Boyd (3)].

Qualitative constancy and rôle of the élément constant.—The qualitative constancy of the *élément constant* in the whole organism, as well as in the tissues, has often been contested. On theoretical grounds Aylward, Channon & Wilkinson consider the constancy of constitution to be in contradiction with the activity of the phospholipins in general metabolism, especially in fat metabolism. But this is not so as Terroine & Belin have expressly declared. According to these authors it is not a question of constancy in the static sense, but rather in the dynamic sense. The constancy is a consequence of two kinds of processes operative in opposite directions; one tends to modify the *élément constant*, the other, which depends on the specific properties of tissues, tends to reconstitute it.

The qualitative constancy of the *élément constant* and more especially of the phospholipins, has been denied by various experiments. Alf Klem, having fed a certain number of animals on fatty substances of high iodine number and another group on fatty substances of low iodine number and having starved these animals to death, found that the fatty acids of the first group had an iodine number of 94.0 and fatty acids of the second an iodine number of 61.5. Diet has also modified the nature of the mixed fatty acids of the *élément constant*. Unfortunately, this affirmation is supported by the results of a technical procedure—extraction by ether, which is not sufficient to remove all of the lipids.

Sinclair, as is known, has energetically denied in former experiments the qualitative constancy of the tissue phospholipins and considered the variability of their constitution to be proportional to their rôle in fat metabolism. He supports this theory by new arguments and points out the mechanism of phospholipin variability. Phospholipins extracted from the whole carcass of rats, which had been maintained on various diets containing fats of markedly different iodine number, yielded mixed acids with an iodine number varying from 104 to 165. The proportion of liquid to solid acids was not variable but variations were observed in the relative amounts of liquid acids; the same results were obtained from tissues such as liver, muscle, kidney, and heart studied separately. Thus we may agree that in the phospholipins, the solid fatty acids are constant, the liquid acids variable [Sinclair (1)]. A diet containing saturated fats does not modify the iodine number of the mixed fatty acids of the organism, but highly unsaturated acids do modify it; with a mixed diet (saturated and unsaturated fats) the same results may be obtained as with unsaturated fats alone. Thus the phospholipins select particularly the non-saturated acids and these acids only. Sinclair, having elevated the iodine number of the mixed acids of the liver to 160 by a diet containing cod-liver oil, observed that this value persisted for thirty-nine days after the diet had been interrupted; thus the phospholipins are able to retain for a long time the highly unsaturated fatty acids obtained from the food [Sinclair (2)]. Sinclair's (3) last investigation shows that elaidic acid can be substituted in the phospholipins of the liver, in the proportion of one-third of all the fatty acids; thus, he admits the existence of two classes of phospholipins: the first contains the highly unsaturated acids which form an essential part of the cell composition; the second, with less unsaturated acids, functions as an intermediary in fat metabolism. These two classes of fatty acids, constitutive and metabolic, are unequally distributed in the different tissues, muscle containing a greater part of the first, liver containing various quantities of both. This eclectic point of view may offer a promise of reconciliation between the still diametrically opposed opinions of Terroine and Sinclair concerning the constancy or qualitative variability of the phospholipins.

ÉLÉMENT VARIABLE

We possess but few new investigations on this element. Timon-David & Ceresola have established that female molluscs contain more fatty acids than male molluscs—a particular example of a general and

well-known phenomenon. They also found that the ethylenic bonds in the lipids are more numerous in the female animals, but the iodine numbers obtained are not at all conclusive, the precision in determination and the observed differences (136.7 against 124.2 in *Murex*, 168.3 against 162.9 in *Eledone*, and 149.3 against 147.5 in *Mytilus*) not being sufficient.

Stolfi (1) is of the opinion that the subcutaneous fat of man is modified with age; the proportion of solid acids increases; the acidity of the total acids varies, but without much regularity; the saponification index falls [Stolfi (2)], and the iodine number rises [Stolfi (3)]. Investigations made on *Pyrocorrhis apterus* have shown that during an annual cycle the total fat value, expressed in per cent of dry substance, increases from June to November (18.52 to 32.70) and thereafter falls to its normal value which is reached in April (Macinca).

Three types of experiments have pointed out the considerable influence of food fat upon the fats of the organism. Lovern (2, 3) shows that the important differences existing in the composition of reserve fat in marine fish and fresh water fish (the latter have more C_{16} and C_{18} acids and less C_{20} and C_{22} acids) is wholly due to the corresponding composition of the fats contained in the plankton utilized by these animals as food.

Platon, Hermansson, Edin & Hansson were able to vary at will the iodine numbers of cow's milk fat by alteration of the food: 45 and 47 with fresh lucerne only, 39 with 72 per cent of lucerne and 28 per cent of grain; 28.5 with 75 per cent of beet root. The modification proceeds very rapidly, as soon as the food passes through the alimentary canal. By feeding rats with oats and maize, Olcott, Anderson & Mendel found an iodine number of the body fat amounting to 79 to 83 and falling to 70 to 71 on feeding with wheat and rye, which are not so rich in fats; with a diet containing maize, the iodine number of the unsaturated acids was 75; with a diet of rye it was 68.5.

Klenk, Ditt & Diebold demonstrate again the influences that temperature may have upon the fatty substances, irrespective of origin, stored in the organism; the proportion of C_{20} to C_{22} unsaturated acids diminishes with increase of body temperature; they form 15 per cent of all the fatty acids of the frog, 7 per cent in the tortoise, 5 per cent in the lizard, and 1.5 per cent in the rat. In this case too, the reptiles are intermediate between poikilotherms and homeotherms. Terroine, Bonnet, Kopp & Véchet had previously made an identical observation in their study of the degrees of saturation of fats.

PHYSIOLOGICAL RÔLE OF THE LIPIDS

PHOSPHOLIPINS

It has been generally thought that the phospholipins have a peculiarly favorable action on nutrition, and physicians have often prescribed them when growth was not satisfactory. The investigations of Pickat, Kurtsina & Zenin do not support this treatment: small doses of lecithin do not promote growth in rats which are well fed and in receipt of the necessary vitamins; but if vitamins A and B are not present in the diet growth is furthered by lecithin, most probably because the administered lecithin is not absolutely free from vitamins. Torrisi, who investigated the goat in lactation, shows that small doses of lecithin (0.4 to 0.8 gm.) increase the body weight, the secretion of milk and, to a small extent, the fat content of milk; but in high doses (1.2 to 2.4 gm.) the flow of milk is considerably diminished; mixtures of lecithin and cholesterol oleate or total egg-yolk lipids were always favorable.

GLYCERIDES, OTHER ESTERS, FATTY ACIDS

The lipids, which have a high potential energy, are employed to regulate the body temperature. Lánčzos reports that the livers of mice which had been subjected to very low environmental temperatures contain more fat (8 to 12 per cent of the fresh weight) than the livers of the control animals (4 to 8 per cent). This storage signifies, most probably, an increased mobilization of the reserve fat to the regions where it will be employed. Evans & Lepkowski consider that the lipids spare vitamin B: the tissues of rats, maintained on a vitamin-B-free diet, are richer in vitamin B if their ration contains a great quantity of fat, than if it contains only a small quantity. Valla concludes, and these deductions seem to us to be very important, that the organism must consume fat, even though needlessly, in its energy expenditure: mice, fed exclusively with abundant quantities of carbohydrate, have a much longer survival than starved animals (twenty-three days for the former, one hundred fifteen hours for the latter, at 30°) but they die also after having lost, like the starved animals, all their fatty reserves. We do not understand this phenomenon.

The much debated question as to whether the glycerides act by themselves or through the fatty acids which they contain, has again been discussed. Lepkowski, Ouer & Evans, who investigated pig fat,

have observed that isolated acids which have again been esterified with glycerol in order to obtain a synthetic pig fat, produce the same effect upon growing rats as natural fat. The isolated fatty acids are only inferior in rations which are very rich in fat; methyl and ethyl esters are equivalent to fats in a ration which contains 25 per cent of fatty substances; but when this proportion rises to 60 per cent these esters, the second especially, prove quite inferior. Lecoq has a somewhat different point of view; he affirms that, if free fatty acids are substituted for the fats of a ration, the animals always have neuritic accidents, and will die even if high doses of vitamin B are added as supplements; glycerol additions are partially, but not completely, amelioratory. The author considers that an essential substance, which ordinarily adheres to fat, is lost while the fatty acids are being prepared; but this point of view is opposed to the fact observed by Lepkowski, Ouer & Evans that natural and synthetic hog fat act alike.

ORIGIN OF THE LIPIDS OF THE ORGANISM AND THEIR PRODUCTS

The origin of the lipids is twofold: from other lipids on digestion, absorption, and conversion, and by synthesis from other foodstuffs.

FORMATION FROM OTHER LIPIDS

Digestion and digestive enzymes.—According to Sure, Kik & Buchanan, the enzymes which are active in mammalian digestion are considerably diminished (77 per cent for oil) on vitamin-free rations. In the pancreatic digestion of oil, studied *in vitro*, Artom & Reale (1) have observed an important increase in the acetyl index and they, therefore, affirm the existence of intermediary products which remain incompletely saponified. While investigating the synthetic activity of pancreatic lipase, they demonstrated [Artom & Reale (2)] an action upon a mixture of oleic acid and glycerol, yielding, in the first place not mono-olein, as Pottevin suggests, but essentially diolein. Gastric juice does not produce any enzymatic hydrolysis of lecithin, but pancreas contains a lecithinase, activated by bile (Jandolo), whose activity consists, most probably, in the splitting-off of the fatty acids.

Absorption.—Intensity of absorption is measured, as is well known, by the coefficient of digestive use. Hilditch considers that it depends almost completely upon the possibility of the fat being liquid at body temperature. Holt, Tidwell, Kirk, Cross & Neale, on the con-

trary, think that the melting point has no influence, in children, upon absorption; fatty acids with one or more double bonds are absorbed more readily than saturated acids. Thus we may conclude, a very important inference, that upon a mixed diet the child absorbs milk lipids of the maternal type with more difficulty than olein, olive oil, or soybean oil. In rats, however, Peretti & Reale have not observed any parallelism between the degrees of unsaturation and the rate of absorption. These authors did not measure the coefficient of digestibility, but only compared the fats still present in the alimentary tract after some time with the fats ingested. But we must admit the possible intervention of factors other than the rapidity of absorption: the rapidity of transit, for instance. Then, in other experiments upon oleic acid, mono- and diolein, Peretti (1) has observed that the slight absorption of oleic acid is but a consequence of the slow evacuation of the gastric contents. Further, Peretti (2) points out that on feeding rats with different fats the amount of fatty acid in the liver increases much more for the alimentary fats of low iodine number (coconut oil and olive oil) than for fats of high iodine value (linseed oil and poppy-seed oil); this is true also for intestinal digestion. But are these experiments reliable? Peretti & Reale (2) show that after ingestion of oleic acid, mono- and diolein, oleic acid gives the highest amount of liver fat, while Peretti (1), on the contrary, had observed that oleic acid was most slowly absorbed from the alimentary tract. These contradictory experiments show that the different factors influencing the digestive use of fats are not yet settled.

Sullivan & Fershtand have studied the rapidity of absorption through the variations observed in the amount of the blood lipids. A maximum was reached in the sixth hour after ingestion of oil by the rat; the initial level was reached three hours later. In the case of subjects with hepatic disease, the increase was very slight. Thus, the liver seems to control absorption.

The nature of the absorbed substances has again been debated. The non-esterified fatty acids in the lymph and the acetyl index of total acetone extract have been determined by Artom & Peretti (3) who confirm the classic opinion: the lipids exist only as triglycerides.

New arguments have strengthened the doctrine which asserts that the phospholipins of the intestinal mucous membrane have a preponderant part in the absorption of fat. These phospholipins are thought to be intermediary products: at first their fatty acids are thought to be displaced by the acids absorbed from the digestion products, and to

form a new substance which varies with the nature of the ingested fats; later this intermediate product gives up to the blood and lymph the fatty acids received from the alimentary tract and then combines with newly absorbed supplies.

New evidence demonstrates that the food fatty acids are really incorporated in the phospholipins of the intestinal mucous membrane, that the absorption of fats depends on a continuous synthesis of the phospholipins and that anything which opposes this synthesis interrupts absorption. As for the first, Artom & Peretti (1, 2) show that the intestinal phospholipins of the rabbit, prepared with absolute purity, contain iodized fatty acids for three days after the ingestion of iodized fat. As for the second, the principal investigations have been made by Verzar and his collaborators. When a rabbit is killed during the absorption of olive oil, the lymph contains considerably more phospholipins and roughly one-sixth of ingested alimentary fats are transported in the lymph as phospholipins. Moreover, a study of the absorption of oleic acid from a portion of isolated rat intestine has shown that the acid is more rapidly absorbed (150 per cent) if glycerophosphoric acid is added (Verzar).

Both these experiments prove that phosphorylation occurs normally and that it is necessary for absorption. In a rat, poisoned by monoiodoacetic acid, fat absorption is completely stopped, both in the intact animal and in isolated intestine; it is well known that monoiodoacetic acid opposes the synthesis of phospholipins. Phlorhizin, which checks the synthesis of phospholipins (Lundsgaard), limits also the absorption of fats [Verzar; Verzar & Laszt (1); Sarzana]. However, it does not suppress, but only diminishes this absorption by a slight degree, 85.9 to 89 per cent as compared with 90 to 92 per cent in the normal subject. Lundsgaard thinks that phlorhizin also has a part in the absorption of glucose for which a phosphorylation is also necessary. Thus the mechanism of carbohydrate absorption is similar to that of the lipids, a fact which investigations of Verzar & Laszt (2, 3) on the action of the suprarenal glands have again demonstrated: in the adrenalectomized animal fat absorption is considerably retarded, but increases in rapidity after injection of a suprarenal hormone, "encorton."

Thus, the suprarenals act by a humoral mechanism on the phosphorylation of both fatty acids and carbohydrates. In order to express the actual state of the problem we may best refer to Verzar's opinion: neutral fats are saponified in the intestine; the fatty acids form with

bile acids a stable complex which may be diffused, resorbed, and carried into the wall of the intestine where it is split; there the liberated fatty acids are integrated into the phospholipin molecule and a synthesis of neutral fat occurs (a process which is still obscure); this fat is taken up by the lymph.

Rearrangement.—Booth, Kon, Dann & Moore report that the absorbing capacity of cow-milk fatty acids, in the region of 230 m μ , increases seasonally when pasturing. Dann *et al.* show that, if cows be fed with fats whose absorbing capacity for radiation of the specified wave length is mean, the fatty acids in the butter fat absorb more strongly than the fatty acids of the food; thus, fatty acids have been converted in the organism. This conversion can be produced *in vitro* by boiling with alcoholic potash and is most probably a molecular rearrangement with the formation of isomeres.

FORMATION FROM CARBOHYDRATES

Lovern (4) investigated two tropical species of carp (region of Singapore), *Hypophthalmichthys nobilis* and *H. molitrix*, fed only with plants containing a mean quantity of fats. He shows that each type deposited fats which were characteristic for the species. Feyder has shown that in the rat the synthesis of fats from carbohydrates occurs more readily from sucrose than from glucose.

FAT TRANSPORT

Mobilization of reserve fat.—Are all fats mobilized in case of necessity or does the organism use them preferentially? Boyd (7), from studies on fever, shows clearly that the organism makes a selection. At the onset of fever the lipid concentration of plasma increases greatly and the iodine number betrays a sudden discharge of non-saturated acids into the blood; as the fever decreases and during recovery the non-saturated acids diminish and the saturated acids increase. Lovern's (1) investigations on salmon, however, give contradictory results: during the fasting period, when the fish migrate and spawn, the deposited fats diminish but in the female no selectivity is evident; in the male there is a slight selection of C₁₄ acids rather than of C₂₂.

Mechanism of transport.—Cahn & Houget have established a complete theory, whose chief elements alone, we can describe: the fatty

acids of the triglycerides, which proceed from the depot fats on liberation by a lipase, become esters of cholesterol and reach the liver; the liver transfers the fatty acids of the sterol to phospholipins, meanwhile desaturating them, and returns them to the circulatory system; they are distributed to the muscles and there the phospholipins are decomposed, the fatty acids are freed and oxidized and glycerophosphoric (or phosphoric) acid is returned to the liver to participate anew in the synthesis of phospholipins. Cahn & Houget support their theory by new analytical investigations on the variability in composition of the blood, liver, and muscle during muscular effort, on hyperthermia, on fasting, and on pancreatic diabetes.

There can be no doubt that lipid transport is accompanied by modifications of the blood affecting elements other than neutral fats, but they do not all confirm the theory just presented. Boyd (7) observes at the onset of fever that all the plasma fats increase but not the esters of cholesterol; when the fever reaches its maximum the proportion of neutral fats still increases, the phospholipins fall to 30 or 40 per cent, esterified cholesterol to 50 or 60 per cent and free cholesterol to 30 per cent. Cahn & Houget's theory concerns essentially the transfer of mobilized reserve fat, but we may well imagine that similar mechanisms operate in the transfer of absorbed fat to the storage places. Terroine has already noted an important increase in cholesterol during digestive hyperlipemia, but did not identify its different forms. Recently, Artom & Peretti (3) observed an increase in the lipid phosphorus of the lymph (which was not necessarily accompanied by an increase in the phosphoaminolipins), and a lowering in the ratio of free cholesterol to esterified cholesterol; in fasting the inversed phenomena occur. Tajima, in investigations on man, shows that the ingestion of 3 cc. of olive oil per kilo brings about an increase of neutral fat from 57.8 to 97.8 per cent, of phospholipins from 20.5 to 25 per cent, and of esterified cholesterol to 29.2 per cent.

Vehicles: blood.—Neither the amount of blood lipids nor their composition vary with age from twenty to ninety years (Page, *et al.*). Normal subjects on a regular diet present only mean variations in their plasma fats during the day [Boyd (6)].

The ingestion of glucose by the rabbit diminishes the amounts of its cholesterol and lecithin, which are said to be cast away by the bile; the amounts of these substances in blood are controlled by the liver with the aid of bile secretion (Asoda).

The amount of fat in the blood of cows (Schoorl) is related

to their butter-fat production; thus, determinations of their blood-lipid content would permit an interesting selection of the best animals for dairy purposes. We must remember that already in 1931 Leroy & Marck were able to affirm, after numerous investigations, "that the lipid value of blood may be considered as the only character which indicates real capacity for butter production."

For the same quantity of ingested fat hyperlipemia is greater in summer than in autumn (Munoz) but it is very difficult to explain this fact. The distribution of free and bound lipids in animals in the fast-ing state, or fed with fatty and non-fatty rations, varies from one subject to another (Reale) and there exists no constant relationship between alimentation and the amount of blood fat.

Relays.—Lovern (1) considers the existence of relays during the transport of fat to be proved: the composition of the fat contained in salmon eggs is quite different from the composition of reserve fat; but, since there is no discrimination or preferential selection from the fats at the moment of release from the depots, we may conclude that a selection is made at some point on the way. The liver, which for a time had been thought to be this relay, has no part in the gestation of *Trygon molacea*, Stolfi (4) having observed that the amount of lipids in liver does not vary with the advance of pregnancy. Boyd (5) thinks that in mammals the placenta has a large part in the transfer of fat from the mother to the fetus.

FAT CATABOLISM

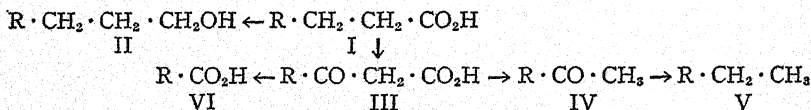
The term "fat catabolism" denotes all the processes by which the lipid character of a substance disappears by total combustion, or by formation of a new, totally different substance.

PRODUCTS WHICH ARE FORMED AT THE EXPENSE OF LIPIDS

Cholesterol.—Cholesterol has often been considered to be related to the fats. Minovici exposes excellently the actual state of this question. He shows, also, that the mouse and the cockroach are able to form cholesterol from oleic acid but not from any other fatty acid. Eckstein, having fed two groups of rats, the first with food containing 11 per cent of fat and the second with food containing 34 per cent, observed that the livers of the second group contained much more sterol than the livers of the first group. As to the origin of cholesterol

from lipids it has often been affirmed that, coincident with the utilization of all body fats, animals starved to death have a greater concentration of cholesterol than normal animals. Valla presumes that this increased concentration in the mouse is not simply apparent, a comparative increase referable to the disappearance of body fat, but that the increase is absolute and very important; the causes of this phenomenon are still obscure. The increase, nevertheless, is surely due in part to a retention of the cholesterol which has been freed by the loss of tissue, and not totally eliminated. According to Valla a cholesterol synthesis is also possible, though not affirmed.

*Hydrocarbons.*¹—Chibnall & Piper have completed their investigations on the constitution of plant and insect waxes. Channon & Chibnall had already found a paraffin, *n*-nonacosane, and its corresponding ketone, *n*-nonacosane-15-one, in the lipids of cabbage leaf. Chibnall & Piper now postulate a mechanism of formation; though a mere hypothesis, it allows an understanding of the appearance of the hydrocarbons and the various constituents of the waxes, proceeding from a small quantity of unsaturated acids with an even number of carbon atoms. The proposed mechanism is as follows: the fatty acid (I) is either reduced to give the primary alcohol (II) or it is oxidized to give the β -keto-acid (III); the latter can be completely modified, either into a methyl ketone with one carbon less (IV) and then into the corresponding paraffin (V), or into an acid (VI) with loss of two carbon atoms through β oxidation. Note that the product (VI) may now serve as a substrate for recurrence of the initial processes:



The variability of the substances issuing from these modifications and the orientation of the reactions are only a consequence of the selective processes which are appropriate to the metabolism of the individual substances concerned.

Vitamins.—Whipple & Church, who fed rats with rations containing but little of vitamin B₁ observed the antiberiberi factor in the feces of these animals; it did not appear when the food contained

¹ Cf. also this volume, p. 110. (EDITOR.)

carbohydrates and proteins. In the alimentary canal of the rat, fats thus have a preponderant part in the formation of the antiberiberi factor.

Carbohydrates.—The question whether carbohydrates in the higher animals may be formed from fats is again under dispute. Gemmill & Holmes consider the formation to be possible and support their opinion by the following facts: sections of rat liver immersed in Ringer's solution, have a respiratory quotient of 0.79 in the normal rat, and of 0.58 in rats which have been fed with fat and whose liver is fatty. The amount of carbohydrate in these fatty liver sections rises in the three hours during which the slices are shaken in Ringer's solution at 37°; finally, rats, whose livers after an appropriate diet cease to contain glycogen, increase their liver glycogen to 1 per cent within four or five days of receiving butter fat in their food. Abelin has shown that a diet exclusively of fat, or a mixed diet containing fat, increases by 40 or 50 per cent the amount of muscle- and liver-glycogen in the rat.

THE LOCUS OF CATABOLISM

A doctrine will ever be successful if supporting a sonorous name, more successful if this name is derived from Greek. This happened to the theory of pulmonary *lipodieresis* of Binet & Roger, who supposed the lung to be the chief site of the conversion of fat. Various assertions, seriously and oft-repeated, give no support to this point of view. Hoppe, Cantoni, Markowitz & Mann, Cacuri, then Kuriyagawa *et al.* deny the fundamental facts which support this theory; whether starved, whether after a fatty meal, no difference at all exists in the plasma and the corpuscles between the blood of the right ventricle and the blood of the left ventricle. If contrary results have been obtained, the technical methods used were probably unsatisfactory, most especially in the introduction of caustic soda not into the right ventricle, but into the vena cava. These authors do not attribute to the lungs any part in normal fat metabolism.

Aylward, Channon & Wilkinson, investigating the livers of rats which had had a fatty meal, find that the amount of glyceride is the same four hours afterward, rises during the six following hours, then falls to normal in the thirteenth hour (these variations corresponding to what occurs in blood); the phospholipins fall during the first seven hours and rise again to normal in the thirteenth hour (Artom finds the phospholipins to increase at exactly the same moment as the other authors find a diminution); the iodine value of the

total fatty acids falls, for both categories of lipids, when the experimental ration contains beef fat.

MECHANISM OF FAT CATABOLISM

Three questions have been studied and debated: Have we initially a desaturation of the fatty acids? Is the theory of β -oxidation exact and can it be used to explain all the phenomena observed? Is the formation of the keto compounds dependent on fat metabolism and what is its mechanism?

Initial desaturation.—We have contradictory opinions concerning this question. Verkade & van der Lee, having themselves ingested triundecylenine, found in their urine a small quantity of sebacic acid which can only appear if undecylenic acid has been split at its double bond. Tesauro has found in placenta a dehydrogenase active on succinic acid but inactive on palmitic and butyric acids.

One of the most important arguments for initial desaturation is the finding, in pig's liver of $\Delta^{12:18}$ -oleic acid which is not to be found in the deposited fats (Hartley). But Channon, Irving & Smith could not obtain this product. They isolated from liver two octadecenoic acids, 85 per cent of which was the ordinary $\Delta^{9:10}$ -oleic acid; the composition of the other acid is not known at present but it is certainly not the acid found by Hartley. The question of initial desaturation is, therefore, still open.

β -Oxidation.—Jowett & Quastel (2) studied the formation of keto compounds by sections of liver acting *in vitro* on different saturated fatty acids with 2 to 10 carbon atoms. They found that acetoacetic acid is formed from the acids of even-numbered carbon atoms and that this formation is more intensive from the higher members of the series than from butyric acid; thus butyric acid cannot be an intermediary. Jowett & Quastel abandon the theory of β -oxidation and propose a new theory, the central element of which is that oxidation occurs at different points on alternate carbon atoms before the linkage splits. Their theory, therefore, is called the theory of "multiple alternate oxidation." Different schemes, which we cannot reproduce here, support this theory.

Formation of keto compounds.—This phenomenon has been studied widely *in vitro* and *in vivo*. Terroine & Trimbach show that a well-balanced and identic alimentation produces in different species (cow, man, pig, cat, dog, rabbit, hedgehog, rat) a ketogenesis which varies with the quantity of food ingested, expressed in terms of unit

body weight. Thus, it corresponds, for different species, to the amount of energy expenditure. Trimbach establishes further, that the same formation occurs with a carbohydrate alimentation, and with any of these diets the excretion of keto compounds is the same as with a mixed alimentation. Thus it is proved that the formation of keto compounds does not depend only on fat catabolism. Trimbach further studied the effects of alimentary substitutions upon different animals receiving a strict carbohydrate diet, and found very different responses in the different species: when fat feeding begins, acetonuria is much more intensive in the rat and the pig than in the dog. In the fasting animal, Trimbach insists once more on the exceptional character of the increased ketogenesis; such increases were never observed in the adult individuals of any species, when fasting followed upon a milk diet.

Jowett & Quastel (3), who studied the mechanism of formation of acetoacetic acid, find that this formation can be observed *in vitro* by the action of different tissues (liver, kidney, spleen, testicle) upon fatty acids. In opposition to Friedmann's opinion, which is generally held, Jowett & Quastel (1) do not regard β -hydroxy-butyric acid as a stage in the formation of acetoacetic acid; they consider that the secondary appearance of the hydroxy acid is possible, though not demonstrated, by reduction of the keto acid. They discuss, without arriving at any precise conclusion, the different hypotheses which may explain the formation of acetoacetic acid.

REGULATION OF METABOLISM

Nervous.—Hausberger, having cut on one side the nerves of the scapulary girdle of the mouse, saw on histologic section that the deposited fats were more abundant on this side than on the other. He concludes—a startling deduction—that the storage of peripheral fat is controlled by the nerves.

Chemical.—Best and his collaborators continue to advance new evidence concerning the influence of choline upon the deposition of liver fat.² Rats, fed with grain and beef fat, undergo an increase in liver fat, the amount rising to 15 per cent within three weeks; if, at this moment, a mixed food is given containing only a small quantity of choline, the liver fat still increases; but if choline is introduced in

² Cf. also this volume, p. 435. (EDITOR.)

the ration in greater quantities (Best & Huntsman) they diminish rapidly. Ingested choline impoverishes of its fat a liver which formerly has been enriched by phosphorus intoxication (Best, Maclean & Ridout). Channon & Wilkinson could only successfully obtain a fatty liver in the rat if they used rations containing at the same time much fat and carbohydrate but little protein; if the food contained fat and abundant protein, the amount of liver fat was unchanged. Proteins can thus be thought of as controlling the formation of fatty liver, perhaps because the organism achieves the synthesis of betaines and choline from certain amino acids. It must be noted that the increase in fat occurs only in the liver, the reserve fat remaining unchanged.

Vitamin.—De Caro & Giani observed that the iodine numbers of the liver and suprarenal fats fall in guinea pigs which do not receive vitamin C in their food. Is this vitamin acting directly and immediately on fat metabolism? New experiments will throw some light on this question.

Hormonal.—Glands of internal secretion (pancreas, thyroid, and hypophysis) have been suggested as having a part in fat metabolism.

Aubertin, Lacoste & Castagnon have observed that, after ligation of the pancreatic duct, a dog presents a picture of fatty degeneration of its liver without any disorder in its carbohydrate regulation. Ralli, Flaum & Banta presume that the liver of the depancreatized dog is richer in fat than normally. In depancreatized dogs receiving pancreas extract in their food, Chaikoff & Kaplan find that the blood fat does not fall as after pancreatectomy, but on the contrary increases above normal both with respect to lipid constituents and cholesterol, whose esterified fraction is lessened in proportion to the free sterol. But the mode of administering the pancreas extract is such that we may not refer these findings to the internal secretion of the pancreas. Insulin given to rats or dogs increases rapidly the amount of glycogen in adipose tissues and increases their water content, but after a certain time these effects disappear. A dog fed with carbohydrates and receiving insulin, grows fat by synthesis of fat from carbohydrates (Baer, Scoz & Boeri).

Schmidt, having administered thyroxin to rabbits, finds that the fatty acids of the liver phospholipins diminish and the nonphosphatidic fatty acids increase; inverse modifications occur in the muscle; both of these categories of fatty acids increase in the blood. It is very difficult to explain these results.

Anselmino, Effkemann & Hoffmann have studied in detail the

regulatory hormone of fat metabolism and think it to be the hormone of the anterior pituitary. They show that this hormone is present in the blood of normal subjects four hours after ingestion of 125 gm. of butter; at this stage of digestion it is the most abundant. We may observe that the serum obtained after injection of glucose, and containing the regulatory hormone of carbohydrate metabolism, when injected into animals diminishes at the same time the total fatty acids of the liver and its saturated acids.

These experiments, however interesting they may be, cannot yet elucidate the obscure question of the hormonal regulation of fat metabolism.

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INSTITUT DE PHYSIOLOGIE GÉNÉRALE DE LA
FACULTÉ DES SCIENCES.
UNIVERSITÉ DE STRASBOURG
STRASBOURG, FRANCE

METABOLISM OF AMINO ACIDS AND RELATED SUBSTANCES*¹

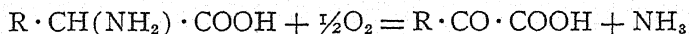
BY H. A. KREBS

*Department of Pharmacology, University of Sheffield
Sheffield, England*

AMINO ACIDS

*Deamination of stereoisomerides² in animal tissues.*³—Tissue slices (kidney, liver) bring about oxidative deamination of both stereoisomerides of the amino acids; the amino acids of the *d*-series, which do not occur naturally, react in many cases much more quickly than the optical antipodes (21, 67, 78). Tissue extracts obtained by grinding the tissue with 10 parts of water deaminate only *d*-amino acids (80). The deamination of *l*-amino acids is inhibited by octylalcohol whereas the deamination of *d*-amino acids is not affected. It may be concluded from these facts that two different systems, "*l*-amino acid deaminase" and "*d*-amino acid deaminase," are responsible for the oxidative deamination of the antipodes (19, 80).

Certain properties of the deaminating systems are of particular interest (66, 80). The *d*-amino acid deaminase can be obtained in clear solutions from acetone-dried kidney or liver. It oxidises, at varying rates, all α -amino acids of the *d*-series which have been tested, with the exception of β -hydroxyglutamic acid. The equation of the reaction is



I

The oxygen uptake is however frequently higher (up to 100 per cent) than would be expected from the amount of ammonia formed, due to a "coupled oxidation" [Keilin & Hartree (66); for details see Keilin (64)]. Substrates which in this way may undergo a coupled oxidation are alcohol (66), hemoglobin (82), and certain derivatives of hemo-

* Received January 17, 1936.

¹ Owing to the stringency of space many topics have had to be omitted.

² The nomenclature used for the optical isomerides is that of E. Fischer and Wohl & Freudenberg.

³ Cf. also this volume, pp. 15, 36. (EDITOR.)

globin (82); the presence of hemoglobin and its derivatives explains the coupled oxidation which occurs in certain preparations of the enzyme. Addition of liver extracts (catalase?) prevents the coupled oxidation (82). The *l*-amino acid deaminase is active in slices or in concentrated suspensions of ground tissue. The activity disappears approximately in proportion to the extent of dilution of the suspension, and this may be explained by the assumption that a ternary collision determines the velocity of the reaction. The probability of a ternary collision decreases in proportion to the dilution (80). The differences in the rates of deamination of *d*- and *l*-amino acids have been confirmed in perfusion experiments by Polonovski, Boulanger & Bizard (117). Experiments on the intact animal do not seem to give clear-cut results owing to various complications. For instance Abderhalden & Tetzner (2) found a small amount of *d*(—) alanine in the urine of a rat after the administration of *dl*-alanine. Such findings, however, give no indication of the relative rates of deamination in the organism, since the body may utilise the natural amino acids without deaminating them. Abderhalden & Tetzner's results therefore suggest that *l*(+) alanine is more rapidly utilised than *d*(—) alanine and are not contradictory to those obtained on isolated tissues or perfused organs. Ample evidence has been accumulated showing that both stereoisomerides of the amino acids can be metabolised by the intact organism [see for tryptophane (16, 17), cystine (55, 134, 141), histidine (34), methionine (60, 135), homocystine (37)].

It seems surprising that the optical antipodes are dealt with by two different enzymic systems and that there is a system which deals specifically with substances which do not seem to occur in nature. Two explanations may be offered for this latter fact: *d*-amino acid deaminase may be an artefact representing some fragment of the *l*-amino acid deaminase. On the other hand, if the synthesis of amino acids primarily yields racemic compounds (which remains to be shown) *d*-amino acid deaminase may be a reagent by which the organism resolves the racemate and obtains the *l*-amino acids.

Site of deamination.—Kidney cortex is the most actively deaminating tissue per unit of weight (80). The activity of liver [see (7)] is about a third of that of kidney, but since the total amount of liver tissue in the body is much greater than the amount of kidney cortex, the share of the liver in deamination is considerable. Moreover the amino acids coming from the intestinal tract reach the liver at a higher

concentration, and a mechanism is thus afforded whereby amino acids of the food can be preferentially deaminated in the liver.

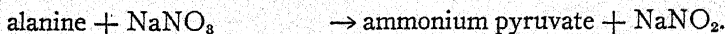
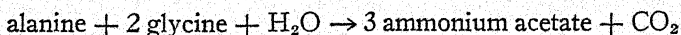
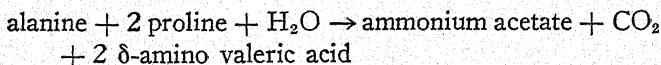
Borsook & Jeffreys (26), using an enzymic hydrolysate of egg albumin as substrate, claim to have found that deamination occurred at about the same rate in liver and kidney. This conclusion is however not valid since liver produces ammonia from tryptic hydrolysates not only by deamination of amino acids, but also by hydrolysis of amides (58, 93, 97) and of histidine (26, 39).

Intestinal wall contains only traces of the deaminating enzymes. If in some experiments [see (92)] a significant deamination of amino acids in the intestine has been observed, this effect is mainly due to the action of bacteria in the lumen as Lauresco (84) has demonstrated. Some of the ammonia which is formed in the intestine may be derived from amino purines or from the fission of amides (asparagine and glutamine) set free during the tryptic digestion of proteins [Terroine & Lauresco (138)]. Muscle tissue (pigeon, frog, frog's heart) has been shown to deaminate some amino acids, though at a comparatively slow rate [Needham (103), Clark, Gaddie & Stewart (33)]. The majority of vertebrate tissues [brain, retina, spleen, testicle, placenta, chorion, red blood cells, bone marrow, pancreas, salivary glands, tumours (80), mammalian heart (34a)] appear to be incapable of deaminating and metabolising amino acids (80). Glutamic acid in certain tissues behaves exceptionally (81, 148) in that it is metabolised. In kidney only the cortex (*Tubuli contorti*) is active (68, 88, 106).

Liver and kidney were found to deaminate amino acids throughout the vertebrate kingdom, and wherever the matter was investigated *d*- and *l*-amino acid deaminases were found together (80).

Deamination in invertebrates has not yet been studied in detail. Lawrie (85) observed a production of ammonia from an unknown source by protozoa.

Bacterial deamination.—Anaërobic bacteria (*Clostridium*) may deaminate certain amino acids oxidatively using as oxidising agents proline, glycine [Stickland (136)], or nitrate [Aubel (8)]:

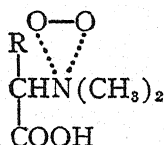


The last reaction is analogous to the oxidation of lactic acid by nitrate, as described by Quastel, Stephenson & Whetham (119).

According to Bernheim, Bernheim & Webster (22) "resting" *Bacillus proteus* can oxidise, deaminate, and decarboxylate various amino acids, molecular oxygen or methylene blue acting as the hydrogen acceptor.

Deamination in model systems.—The study of the oxidative breakdown of amino acids in model systems still seems to be of biological interest, if the conditions and reagents are not too remote from those occurring naturally. Whether the deamination ("aminolysis") brought about by heating amino acids at high temperature with charcoal or charcoal extracts (13, 14, 15, 151) or the interaction between amino acids and glucose at 130° [Akabori (5)] will prove of immediate biological interest remains to be shown.

Bergel & Bolz (18a) report that the N-dialkyl amino acids are much more readily oxidised on charcoal than are the corresponding amino acids. N-dialkyl amino acids are also autoxidisable in the presence of hemin, whereas amino acids are not. The main end products of the oxidation in these models are aldehyde, carbon dioxide, and amine. The primary product—and this is of special interest—is probably a peroxide:

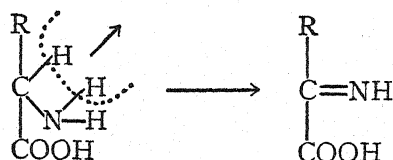


The existence of such peroxides and their formation in the photochemical autoxidation of amines has been proved by Gaffron (48). The formation of a peroxide in the model is of especial interest, for a peroxide may be the primary product of the biological oxidation (see the following section).

Mechanism of deamination.—The following facts are relevant to the mechanism of the deamination:

1. Keilin & Hartree (66) studying the deamination of methylated amino acids by *d*-amino deaminase found that *d*()N-monomethylalanine is deaminated where *d*()N-dimethylalanine and *d*() α -amino isobutyric acid are not attacked; see also Krebs (77). This is in agreement with the scheme put forward by Wieland & Bergel

(150) and by Knoop & Oesterlin (73), who studied the behaviour of methylated amino acids in model systems. The primary step in deamination is thought to be the removal of two hydrogen atoms and the formation of the unsaturated imino acid:



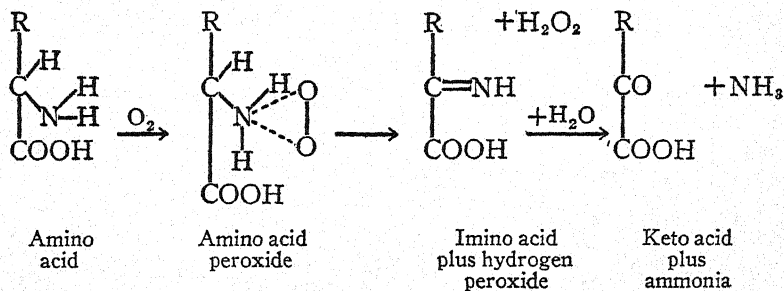
II

It is obvious that the substitution of either of the two hydrogen atoms involved makes reaction II impossible.

2. Keilin & Hartree's (66) observations that a coupled peroxidative oxidation occurs if suitable substrates [alcohol (66), hemoglobin (82)] are added to *d*-amino acid deaminase suggests that a peroxide (hydrogen peroxide) is formed.

3. Bergel & Bolz (18a) demonstrated the formation of a peroxide when methylated amino acids are oxidised in model systems (see the previous section).

These three observations allow, in the view of the writer, the following tentative formulation for the intermediate steps in deamination:



III

Bergel & Bolz showed that the dehydrogenation (II) is not a necessary step in the oxidation on charcoal since *N*-dimethyl-amino-isobutyric acid which has no hydrogen available is oxidisable to

acetone, carbon dioxide, and dimethylamine. In this case the end products arise more or less directly from the amino peroxide without the intermediation of the imino acid.

Scheme III refers only to *d*-amino acid deaminase; the deamination of *l*-amino acids may well take a different course.

Diamino acids.—In the previous sections the deamination of the α -amino group only has been dealt with. The deamination of the ω -amino group of the diamino acids has not been studied in detail. As would be expected, the *d*-amino acid deaminase deaminates only the α -amino group of *d*(—)lysine and *d*(—)ornithine (82).

*Primary steps in catabolism.*⁴—Deamination is the primary step in the catabolism of the simple α -amino acids, of the α -amino-dicarboxylic acids and of all the amino acids of the *d*-series. In the case of amino acids of a more complicated structure, before deamination takes place, other groupings of the molecule may be attacked. As primary steps the following may occur:

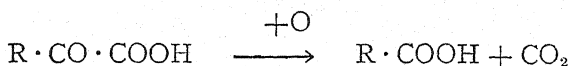
histidine	→ cleavage of the imidazole ring under the action of histidase [Edlbacher & Neber (39)],
tryptophane	→ ring splitting and formation of kynurenine and kynurenic acid [Kotake (75)],
methionine	→ demethylation [Brand <i>et al.</i> (29, 30)],
sulphur-containing amino acids	→ oxidation of the sulphur to sulphate (?) [see (115)],
phenylalanine	→ oxidation to tyrosine [see Edson (41)],
tyrosine	→ oxidation of the ring [see Bernheim (19)].

It is probable that these pathways are open to the natural *l*-amino acids only. Since deamination is the primary reaction in the *d*-amino acids, it becomes clear why antipodes give, in certain cases, different end products [tryptophane (17)].

Fate of the ketonic acids; ketogenesis from amino acids.—The fate of the α -ketonic acids formed by deamination is not completely known. Various pathways are possible: (*a*) reduction to the hydroxy acid; (*b*) resynthesis of the amino acid (see page 253); this may play a rôle in the conversion of foreign amino acids into the optically

⁴ The statements made in this section refer to the tissues of the higher animals. Many other reactions occur in bacterial metabolism.

active natural isomerides; (c) breakdown to the fatty acid with a carbon chain shorter by one carbon atom:

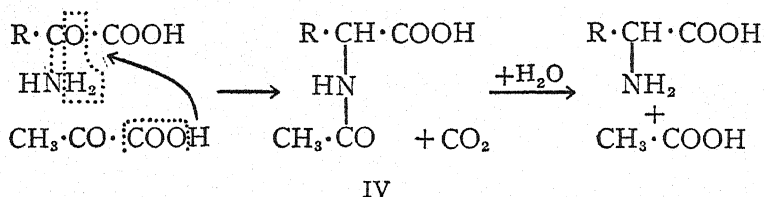


The question is still open whether the decarboxylation is the first step and the oxidation of the aldehyde the second, or whether both reactions ("oxidative decarboxylation") occur simultaneously. It is more probable that oxidative decarboxylation is the mechanism which occurs in the animal body (as distinct from yeast cells) because animal tissues do not contain any significant amounts of carboxylase. Moreover, aldehydes yield in liver only 50 per cent carboxylic acid, the other 50 per cent being reduced to alcohol. This dismutation of aldehydes, discovered by Parnas (108) and Batelli & Stern (11), has recently been studied by Reichel *et al.* (121).

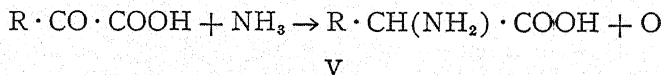
Edson (41), using the tissue-slice method, reinvestigated the ketogenesis which occurs when amino acids are metabolised. The most strongly ketogenic amino acids were found to be leucine, tyrosine, and phenylalanine (rat liver). This is in accordance with the perfusion work of Embden *et al.* (43). The only other amino acid which proved considerably ketogenic was hydroxyproline. Of the other amino acids some are slightly ketogenic, but this effect seems to be due to the ketogenic effect of ammonia which is liberated from amino acids by deamination. That the presence of ammonia increases the formation of acetoacetic acid was first demonstrated by Annau (6) who found that pyruvic acid yields acetoacetic acid in the starved liver if ammonia is present. Edson (40) studied this ammonia effect in detail and showed that a number of previous experiments on ketogenesis have been wrongly interpreted since this effect was not known. Embden *et al.* (43) and Hensel & Riesser (56) neutralised their substrates frequently with ammonia and found an increased yield of ketone bodies when phenyl lactic acid or muconic acid were perfused. Edson (41) confirmed the observations, but showed that ammonium chloride has the same ketogenic effect whereas the sodium salts of phenyl lactic acid and muconic acid do not yield acetoacetic acid. These results are of interest for the intermediate metabolism of tyrosine and phenylalanine (see pp. 256, 257).

Synthesis of amino acids.—Neber (102) showed that surviving slices of rat liver form large quantities of amino nitrogen when am-

monium pyruvate is added. This seems to confirm Embden's perfusion experiments on the synthesis of alanine from ammonium pyruvate. The writer (82) is able to confirm Neber's experiments for rat and bird livers, but he finds no amino nitrogen formed when pyruvate is replaced by other ketonic acids, e.g., ketoglutaric acid. This lends support to the idea proposed by Knoop (70, 72) long ago that pyruvic acid may play a special rôle in the synthesis of amino acids in such a way that condensation between ketonic acid, ammonia, and pyruvic acid is the primary step, leading to an acetyl amino acid according to the following scheme:



This hypothesis was based on: (a) the isolation of the acetyl amino acids (70, 105) from urine and tissues: (b) the fact that acetyl amino acids can be utilised in the organism (16, 70); and (c) the well-known analogous reaction *in vitro* (44a, 61). It was, however, abandoned by Knoop & Blanco in 1925 (71), but revived by du Vigneaud & Irish (142) who claim that Knoop's argument in favour of abandoning the hypothesis was based on a misconception with regard to the special configuration of the acetyl amino acids concerned. Strong support for the scheme lies in the fact that the formation of the amino acid does not require the presence of oxygen (82, 102). Since in the absence of oxygen there is no significant source of energy in the liver, the synthesis of amino acids according to the reaction

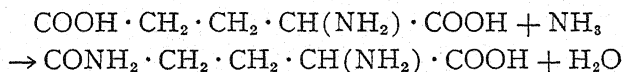


must be formulated as an exothermic process. Scheme IV complies with this condition; the oxygen arising from the reduction of the ketonic acid according to reaction V is used for the oxidation of pyruvic acid to acetic acid, and the net change of energy in this coupled oxido-reduction is a loss of free energy. Further evidence

is the fact that the formation of acetic acid, which must be a by-product according to scheme IV, has actually been demonstrated by the work of Annau (6) and Edson (40) on acetoacetic acid formation in the presence of ammonium pyruvate. Acetic acid would be expected to appear under the given conditions as acetoacetic acid.

Glycine.—The relations of glycine to creatine [see Rose (124)] have been further studied by Adams, Power & Boothby (4). The finding of Weber (146) that patients suffering from pseudohypertrophic muscular dystrophy show an increased excretion of glyco-cyamine (guanidino acetic acid) when glycine is administered has been confirmed by Thomas (139). Weber found glyco-cyamine also in normal urine (147) and this, according to Weber, lends new support to the hypothesis that glyco-cyamine is a normal intermediate in a conversion of glycine into creatine [see the discussion of the problem in Rose's review (124)].

Glutamic acid.—Glutamic acid has been shown to play a special rôle in the metabolism of nervous tissue (81). Grey cortex and retina are capable of binding large quantities of ammonia (up to 0.8 per cent of the tissue dry weight per hour), if glutamic acid is present (81). The reaction by which ammonia is bound is the synthesis of glutamine:



No other amino acid has been found to react similarly. The synthesis of glutamine was first found in the kidneys of guinea pig and rabbit which can bind ammonia in quantities amounting to 1 to 2 per cent of the tissue dry weight per hour. The kidneys of sheep and rat synthesise glutamine to a less extent and the kidneys of cat, dog, pig, and pigeon are not capable of synthesising glutamine. Systematic examination of other tissues shows that the system which synthesises glutamine occurs in the grey matter of the nervous system and in the retina of all the vertebrates investigated, but it is absent from the other tissues with the exception of the kidneys of some of the animals mentioned above. The synthesis of glutamine is, under physiological conditions, an endothermic reaction. The energy required is derived from respiration in kidney and brain and from glycolysis in retina. As would be expected the synthesis is bound up with the cell structure since a transmission of energy is involved. Aqueous extracts

from those tissues which synthesise glutamine contain an enzyme which reverses the synthesis and splits glutamine into glutamic acid and ammonia. This enzyme, "glutaminase," is specific for glutamine; it does not split asparagine or glutamine peptides. There is evidence in favour of the assumption that "glutaminase" is a fragment of the synthesising systems and that under physiological conditions it is concerned only with the synthesis. The identity of the synthesising enzyme present in the tissue and the splitting enzyme found in extracts is indicated by the fact that synthesis and splitting are equally inhibited by the foreign *d*(—)glutamic acid. "Glutaminase" is, moreover, inhibited by the natural *l*(+)glutamic acid and this inhibition is competitive. That the splitting enzyme plays a physiological rôle is furthermore rendered questionable by the fact that the pH optimum is 8.8 and that the activity is very low at physiological pH values.

In liver, a second "glutaminase" has been found (81); this glutaminase is not inhibited by glutamic acid and its pH optimum is 7.5; it is probably concerned with the hydrolysis of glutamine derived from the digestion of proteins. Liver glutaminase, too, is distinct from asparaginase (81).

A synthesis of glutamine (and asparagine) from ammonium salts and the amino acid has long been known to occur in plants. Recent contributions to this problem are the papers 51, 129, 140.

The physiological rôle of the synthesis of glutamine in nervous tissue is obscure. It is of interest that glutamic acid and glutamine have some striking effects on oxidations and on lactic acid production in those tissues in which the system occurs [for details see Weil-Malherbe (148)]. According to Tatum *et al.* (137) the presence of glutamine or asparagine seems to be necessary for the fermentation of starch by certain butyric acid bacteria.

Tyrosine.—Bernheim (19) states that *l*(—)tyrosine is oxidised by chopped liver in such a way that one molecule of tyrosine takes up four atoms of oxygen. Edson (41) was unable to obtain constant results for the additional oxygen uptake under the conditions stipulated by Bernheim & Bernheim. Yet it is certain that tyrosine is metabolised in liver and that acetoacetic acid is formed. *p*-Hydroxyphenylpyruvic acid and homogentisic acid also yield acetoacetic acid and are thus possible intermediates in the catabolism of tyrosine. The stages between homogentisic acid and acetoacetic acid are not known. The view that muconic acid is an intermediate (56) has been disproved since this substance does not yield acetoacetic acid. The con-

trary results of Hensel & Riesser (56) are explained by Edson (41) as due to the ammonia effect (see page 261).

Thyroxine, di-iodotyrosine, thyronine.—The biochemistry of these substances has been recently reviewed by Harington (53).

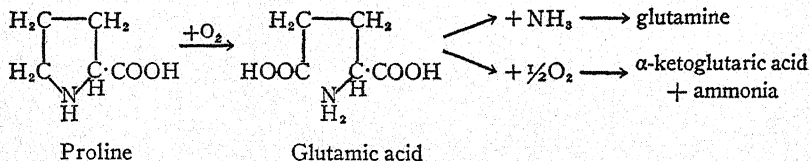
Phenylalanine.—Phenylalanine yields acetoacetic acid in liver whereas phenylpyruvic and phenyl lactic acids do not form ketone bodies [Edson (41)]. This proves that these acids are not the intermediates on the main pathway of the breakdown of phenylalanine in liver. On the other hand, phenylpyruvic acid can certainly arise from phenylalanine in kidney slices (78). That this may also occur in the intact organism is shown by the fact that phenylpyruvic acid is excreted when phenylalanine is fed to rabbits (76, 130), and that it may occur in human urine in a pathological condition described by Fölling (46) as *Imbecillitas phenylpyruvica* [see also Penrose (114)]. There must be, therefore, two different catabolic paths for phenylalanine. The path *via* phenylpyruvic acid may be the preferential path in kidney. As to the path in liver the evidence points to the view that tyrosine is the intermediate [Edson (41)]. Tyrosine yields the same end products, namely acetoacetic acid and, in alcaptonuric patients, homogentisic acid; Embden (43) has demonstrated in perfusion experiments that phenylalanine is converted into tyrosine by the liver and Medes (95) has described a case of an inborn error, "tyrosinosis," in which administered phenylalanine is excreted in the urine as tyrosine.

Tryptophane.—The intermediate metabolism of tryptophane has been exhaustively reviewed by Kotake (75) and only a few papers need be mentioned here. Gordon & Jackson (49) synthesised *dl*-amino N-methyl tryptophane, Bz-3-methyl tryptophane (β -5-methyl indole α -amino propionic acid), and Bz-2-methyl tryptophane (β -2-methylindole α -amino propionic acid) and found that only the first substance was capable of stimulating growth in rats subsisting on a diet deficient in tryptophane. The experiments on the deamination of the N-alkyl amino acids (see page 250) explain this observation. In the same way as *d*-N-methyl alanine is converted to pyruvic acid, *d*-amino N-methyl tryptophane will be converted into indolepyruvic acid which is known to be capable of replacing tryptophane in the diet (18).⁵

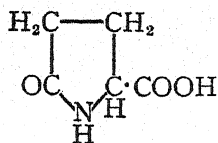
⁵ The experiments of Fishman & White (45a) showing that *dl*-amino-N-methyl histidine may replace histidine in the diet can be explained similarly.

Woods (153) has shown that tryptophane is quantitatively converted into indole if thick suspensions of *Bacillus coli* are continuously aerated. One molecule of tryptophane requires five atoms of oxygen for its oxidation to indole and the side chain is completely oxidised to carbon dioxide, water, and ammonia. The course of the reaction is unknown. The following indole derivatives did not yield indole and are therefore not intermediates: β -indole aldehyde, β -indole carboxylic acid, β -indole acetic acid, β -indole propionic acid, β -indole acrylic acid. β -indolepyruvic acid gave rise to a 10 per cent production of indole, but only in the presence of ammonia. Under anaërobic conditions, indolepropionic acid was formed by the bacteria. Hapold & Hoyle (52) showed that indole formation occurs also in suspensions of *Bacillus coli* which have been killed by chloroform treatment.

Proline.—Bernheim & Bernheim (20, 21), who first described the oxidation of proline in chopped liver, did not isolate the end products. Krebs (78) found that the rate of oxidation of proline is much greater in kidney than it is in liver. Weil-Malherbe & Krebs (149) found an increase in the concentration of amino nitrogen during the oxidation of proline. The amino compound, however, undergoes further decomposition and cannot be made to accumulate in quantities sufficient for isolation. It was possible, however (149), to isolate α -ketoglutaric acid and ammonia when the tissue (guinea pig and rabbit kidney) was poisoned with $10^{-3} M$ As_2O_3 and this shows that the amino compound formed from proline is glutamic acid. As_2O_3 inhibits the oxidation of ketonic acids to a greater extent than it inhibits the oxidation of amino acids and is therefore suitable for checking the oxidation of amino acids at the stage of the ketonic acids. The formation of glutamic acid from proline was also indicated by the formation of glutamine when proline and ammonium salts were added to kidney. The oxidative breakdown of proline may thus be formulated in the following way:



The intermediate stages between proline and glutamic acid are unknown. Pyrrolidone carboxylic acid,



is not an intermediate since it is not metabolised under conditions under which proline reacts (149).

Hydroxyproline.—Hydroxyproline yields in guinea-pig kidney, if ammonia is present, small amounts of an amide which behaves like glutamine. The quantities of amide are much smaller than those formed from proline (149). The formation of glutamine may be explained on the assumption that some hydroxyproline is primarily reduced to proline, but the bulk of the hydroxyproline seems to be catabolised by other unknown reactions. Hydroxyproline increases the formation of acetoacetic acid in liver whereas proline has no effect [Edson (41)].

Histidine.—Kapeller-Adler continued her studies on the excretion of histidine during pregnancy. This histidinuria is found only in human subjects (62). The excretion begins in the fifth week of pregnancy and ceases three days after birth. The quantities excreted depend largely on the diet and added histidine is almost completely excreted. The livers of women dying during pregnancy did not, with one exception, contain active histidase whereas control livers contained the enzyme. The absence of active histidase is, according to Kapeller-Adler (62), the cause of the histidinuria. Such an inactivation of histidase during pregnancy would ensure an adequate supply of histidine to the fetus.

Sulphur-containing amino acids.—The observations of Brand *et al.* (29, 30) on cystinurics have substantially advanced the knowledge of the intermediary metabolism of the sulphur-containing amino acids. This work, in conjunction with other investigations, has established the fact that cystine (29, 30, 57), homocystine [bis (γ -amino- γ -carboxy propyl) disulphide (see 29)] and glutathione ($-\text{SH}$) (30, 55) are almost completely oxidised by cystinurics, whereas cysteine (30), homocysteine (29), and methionine (30, 89) are excreted largely as additional cystine.

These facts lead to the following conclusions [Brand *et al.* (29, 30)]: (a) Cystine can be catabolised without previous reduction to cysteine. (b) Glutathione ($-\text{SH}$) can be catabolised without pre-

vious hydrolysis, which shows that the metabolic behaviour of an amino acid may depend upon whether it is catabolised as the free amino acid or in combined form as a peptide. (c) One of the pathways of methionine catabolism seems to be its conversion into cysteine. All the evidence is in agreement with the assumption that a demethylation to homocysteine is the first step in this conversion [see also Pirie (115), Dyer & du Vigneaud (37), and page 264 of this review].

Medes (96) reports that cystine sulfoxide ($C_6H_{12}O_6N_2S_2$) is oxidised in the body to sulphate and can replace cystine in the diet. This lends support to the theory that the compound may be an intermediate in the metabolism of cystine. Robbers (123) found that decarboxylated cystine and cysteine, cystamine and cysteamine are metabolised when injected into a dog.

AMMONIA

Tissue ammonia.—All excised animal tissues which show metabolic activities at all are capable of forming some ammonia (36, 78), but the precursors of this ammonia are not all known. Amino acids are the main precursors in liver and kidney and may also form some ammonia in the intestine (92) and in the frog's heart (33). In muscle, adenylic acid is the preferential, though not the only source [Parnas (111)]. In sheep blood it accounts for 60 per cent of the ammonia which can be formed (54) and in brain for about 75 per cent (122). The rôle of glutamine as parent substance of ammonia in brain and retina remains to be investigated (see page 255).

Urinary ammonia.—This subject has been thoroughly reviewed by Schneller (127).

Certain herbivores (rabbit and guinea pig) are capable of storing considerable amounts of ammonia as glutamine in their kidney, but it is an open question whether this reaction is of importance for the formation of urinary ammonia (81). The main precursors of urinary ammonia are amino acids. The extent to which amino acids are deaminated is not governed by the acid-base balance. The pH optimum for the deamination of amino acids lies around pH 7.4 (80) whereas the excretion of ammonia increases with decreasing pH of blood and urine. The kidney may produce more ammonia than is needed for the neutralisation of excreted acids and the excess leaves the kidney with the venous blood (101). Whether the ammonia formed

in the kidney is excreted or appears in the venous blood depends on the condition of acid-base equilibrium (80). That ammonia formation is independent of ammonia excretion has also been demonstrated by Polonovski, Boulanger & Bizard (118).

The view that urea is a precursor of urinary ammonia (25) has again been disproved (117).

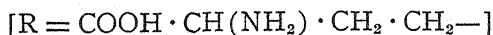
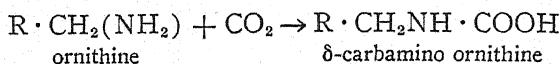
Utilisation of ammonia.—Reactions by which ammonia can be utilised and converted into organic compounds are: formation of urea, of uric acid, glutamine (see page 255), asparagine, amino acids, and the reamination of inosinic acid. The latter reaction is still entirely obscure and one of the outstanding problems in this field.

Effects of ammonia on tissue metabolism.—According to Annau (6) and Edson (40) ammonia increases the rate of acetoacetic acid formation in liver, especially if pyruvic acid is added as substrate. This effect may be explained by reaction IV, according to which pyruvic acid is converted into acetic acid which latter substance is known to yield acetoacetic acid. Ammonium chloride also exerts an effect on the oxygen uptake of tissues and this effect is remarkably different in different tissues (40). Ammonium chloride, 0.01 *M*, does not affect the respiration of brain or spleen but it inhibits the respiration of liver by 20 per cent or more. If glucose or lactate are present it increases that of kidney by about 50 per cent, an effect which is not accompanied by a disappearance of ammonia.

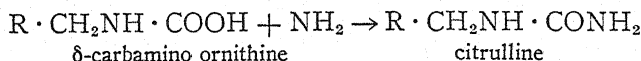
UREA

Urea synthesis from ammonia.—The specific effect of ornithine in increasing the rate of urea synthesis has been confirmed by Borsook & Keighley (27) and by Neber (102), using the tissue-slice method. London *et al.* (91), using the method of angiostomy, reported experiments which, in the view of the writer (79), demonstrate the effect of ornithine in the intact animal.

The rate of urea synthesis increases rapidly with increasing concentrations of the bicarbonate- CO_2 -buffer of the medium. There is almost no synthesis if the carbonic acid buffer is replaced by phosphate (83), whereas the reaction proceeds almost normally in the absence of other ions (83). This specific effect of the bicarbonate buffer may be explained on the assumption that the first stage in the urea synthesis is the formation of the carbamino compound of the δ -amino group of ornithine according to the equation:



Citrulline, the next stage, is the acid amide of the carbamino acid of ornithine:



This view is supported by measurements of the dissociation constant of δ -carbamino ornithine [Roughton & Krebs (125)].

According to Borsook & Jeffreys (26) the net change of free energy in the synthesis of urea from ammonia, acid, and carbon dioxide is, for the conditions existing in the plasma, +14,300 calories.

Enzymes of the urea synthesis.—The first stage in urea synthesis, the formation of the δ -carbamino ornithine, appears to be a non-enzymic reaction. The formation of citrulline and arginine require complex enzymic systems, which cannot be separated from the structure of the living or surviving cells; they are destroyed by grinding the tissue and no details are known about these enzymes. The last step in the synthesis, the hydrolysis of arginine under the influence of arginase, is an enzymic reaction of usual type.

Parnas (109) has pointed out that ornithine may be looked upon as playing the rôle of a coenzyme in urea synthesis, in that it is a heat-stable, crystalloidal component of the urea-synthesising system. It is by itself catalytically inactive, but it becomes active through an additional factor, "the enzyme," and it furnishes the prosthetic group of the complete system. The nature of the enzyme and the mechanism by which it reacts with the "coenzyme" remain to be studied; but experiments on the influence of the concentration of ornithine on the rate of urea synthesis indicate that the enzyme and ornithine form a dissociating compound (82). The recent work from Parnas' laboratory (110, 111, 112) on the rôle of the coenzyme (adenosinetriphosphate) in lactic acid and alcohol fermentations and the work of Warburg's school (145) on the coenzyme in the hydrogen-transferring systems are of great interest in connection with this problem.

Urea production in various tissues.—Botella-Llusia (28) claims to have found a disappearance of ammonia and a formation of urea in

surviving human placenta (40 mg. of urea N per 100 gm. per hour). Franken & Krebs (47), however, were unable to find a utilisation of ammonia in this tissue; traces of urea which may be formed by placenta and the majority of tissues (36) are derived solely from arginine (126). Klisiecki (69) analysed the urea concentration of the blood in the coronary sinus and the left ventricle and calculated from the difference in the concentrations and the rate of blood flow the production of urea by the dog heart. This was found to be enormous, amounting to 4 gm. of urea per twenty-four hours for a heart of a dog weighing 14 kg. Since, however, the same author reports that mere defibrination of the blood caused a marked rise in its urea content the significance of these figures remains doubtful. According to Clark *et al.* (33) urea is, however, produced by the frog's heart.

When urea is formed by tissues it is essential to distinguish between synthesis from ammonia and hydrolytic formation from ureides. The synthesis has been shown in liver only, but hydrolytic formation may arise in most tissues from arginine. In addition it may arise from canavanine in liver, from uric acid, allantoin, and allantoic acid in amphibian and selachian tissues and in many plants (see 83), and from guanidine in moulds (32, 59).

URIC ACID

Uric acid will be dealt with in this article only insofar as it is the main nitrogenous end product of protein metabolism in the uricotelic organisms. Needham (104) has studied the uric acid content of the nephridia and other tissues of a large number of molluscs. Assuming that uric acid will be retained if the metabolism is uricotelic, he confirmed the view [Delaunay (35)] that this form of metabolism is an adaptation to terrestrial life.

Baldwin (10) claims that he found an increase of uric acid production by slices of the hepatopancreas of *Helix pomatia*, when tartaric acid and urea were added. He suggests that in snails uric acid may arise from these two substances according to Wiener's scheme, which, however, has been disproved for uric acid synthesis in birds. Baldwin suggests further that urea is synthesised in *Helix* by means of the "ornithine cycle." This hypothesis would explain why arginase is present in exceptionally large amounts in uricotelic gastropods [Baldwin (9)].

Edson, Krebs & Model (42) showed that hypoxanthine is an intermediate in the synthesis of uric acid from ammonia in the avian

organism. In some birds, such as the pigeon, the synthesis of uric acid requires, besides the liver, another tissue. The liver performs the primary steps in which ammonia is bound, but the end product in liver, as shown by isolation and analysis, is hypoxanthine. This substance is further oxidised to uric acid under the influence of xanthine oxidase which is present in the livers of some birds (fowl), but missing in the pigeon liver [Morgan (99)]. This explains why fowl liver may form uric acid whereas, in the case of the pigeon, other tissues such as kidney or pancreas which are known to contain xanthine oxidase (99) have to act in addition. Schuler & Reindel's (128) observations also made probable the formation of a "purine" in pigeon liver, which was, however, not identified.

AMINES

Wacek & Löffler (143) describe micromethods for the determination of volatile amines in biological fluids using for identification and assay the characteristic crystals which amines form with nitronaphthols. In normal urine between 0.5 and 4 mg. per cent of dimethylamine were found, whereas mono- and trimethylamine only occurred in traces. In two cases with severe burns the amounts of trimethylamine in the urine were enormously increased. Observations by Kaunitz & Wacek (63) and Wacek & Raff (144) suggest that trimethylamine and allylamine may occur in pus and Eppinger *et al.* (44) point out that amines may play an important rôle in the pathogenesis of inflammatory processes.

Lintzel (90) found, in addition to traces of trimethylamine, considerable quantities of trimethylamine oxide in human urine (37 mg. N per day). After a meal of 400 gm. of herring the figure increased to 145 mg. of amine nitrogen per day. Trimethylamine given *per os* is excreted mainly as the oxide. Betaine or choline did not significantly increase the amine excretion.

INBORN ERRORS

Cystinuria.—The work of Brand and his collaborators (29, 30) on the intermediary metabolism of sulphur compounds in cases of cystinuria has been mentioned on page 259. The cystine excretion in cystinuria is caused mainly by dietary methionine, but the inborn error is concerned, according to Brand *et al.* (29) with the metabolism of cysteine. The experiments reported on page 259 show that

in cystinuria there is no failure to demethylate methionine or to convert homocysteine into cysteine.

Hickmans & Smallwood (57) confirmed the increase on standing in the cystine content of cystinuric urine and assume that cystinurics excrete a precursor of cystine which later decomposes with the liberation of free cystine. Brand *et al.* (29) point out that the observed phenomenon could also be due to the gradual disappearance from urine of a substance [ascorbic acid (?)] which inhibits colour development in Sullivan's reaction.

A case of canine cystinuria described by Morris, Green, Dinkel & Brand (100) will be of great interest for future work in this field. The sire and the dam of this dog are registered with the A.K.C. and this will greatly facilitate the investigation of this family of dogs and may permit the breeding of a special cystinuric strain. Cystinuric dogs will be of equal interest to workers in the fields of genetics and of intermediary metabolism.

Imbecillitas phenylpyruvica.—Two more cases of this anomaly [Fölling (46)] have been described by Penrose (114). Edson (41) points out that the error may be due to a failure to convert phenylalanine into tyrosine although a block in an alternative path is also possible.

SOME CLINICAL ASPECTS

The considerable advances in our knowledge of amino acid metabolism already recorded have so far given few decisive results in the field of practical medicine. A few points may be briefly discussed.

Histidine therapy of peptic ulcers.—Parenteral injection of histidine hydrochloride has been recommended for the treatment of gastric and duodenal ulcers (1, 23, 24, 31, 38, 133, 152). The fact that amounts of histidine which are of the same order as the quantities present in the proteins of food may have a therapeutic effect when administered parenterally could be explained since histidase in the liver will act on the histidine derived from food, decomposing it before it can reach other tissues.

Effect of tyrosine on experimental hyperthyroidism.—Abelin (3) demonstrated a favourable effect of *L*(—)tyrosine similar to the effect of di-iodotyrosine on the symptoms of rats fed with thyroid. Basal metabolism, weight, glycogen content of liver, and rate of respiration tended towards the normal when 0.1 to 0.7 gm. of tyrosine per day were fed.

Liver-function tests.—The question whether the concentration of amino nitrogen (87, 107, 113, 120, 131, 132) or ammonia (74, 86, 98) in the blood under various conditions, especially after the intake of amino acids, may be used to indicate the degree of damage to the liver in pathological conditions has again received much attention. Although the results obtained in these investigations may prove of general interest, the value of such "functional tests" for clinical purposes remains doubtful.

Reviews on the following subjects have appeared:

Metabolism of proteins and amino acids [Luck (94), Felix (45)]; metabolism of ammonia [Schneller (127), Polonovski (116), Delaunay (35)]; metabolism of tryptophane [Kotake (75)]; thyroxine and related substances [Harington (53)]; inborn errors and other anomalies [Gottschalk (50)].

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DEPARTMENT OF PHARMACOLOGY
UNIVERSITY OF SHEFFIELD
SHEFFIELD, ENGLAND

MINERAL METABOLISM*

By E. B. HART AND C. A. ELVEHJEM

*Department of Agricultural Chemistry,
University of Wisconsin, Madison*

The mineral elements of biological significance have not only increased in number during the past few years, but new and more important functions have been ascribed to some of the elements which, for a long time, have been known to be essential.

Certain phases of the subject have been treated by Shohl.¹ The metabolism of calcium, magnesium, and phosphorus has recently been reviewed by Schmidt & Greenberg (1), and the biological significance of copper has been summarized by Elvehjem (2). We will confine our discussion to the more recent papers on iron and copper, to manganese, zinc, cobalt, nickel, fluorine, iodine, bromine, selenium, and to brief mention of boron, arsenic, aluminum, and silicon.

IRON AND COPPER

Practically all workers are now agreed that copper is necessary as a supplement to iron for hemoglobin formation in red-blooded animals, and that of a large group of elements copper is unique in this respect. It is also definitely established (3, 4, 5) that copper is not concerned with iron assimilation but functions in the conversion of the absorbed iron into hemoglobin. Recent studies have been concerned mainly with the mechanism by which copper functions in the actual formation of hemoglobin [reviewed by Elvehjem (2)] and the availability and assimilation of the iron in foods and iron compounds. The original conclusion of Bunge that the iron in our food is present only in complicated organic form and that this form of iron is absorbed and assimilated for hemoglobin production must be revised completely. The inability of animals to utilize hematin iron was first demonstrated in 1929 [Elvehjem (6)]. Recent papers by Elvehjem, Hart & Sherman (7) present data to show that all food materials contain two forms of iron, one which reacts with bipyridine under the conditions described by Hill (8) and the other, hematin iron, which does not react with this reagent. The non-hematin iron

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¹ *Ann. Rev. Biochem.*, 2, 207 (1933) ; 3, 209 (1934).

is the only form which is available to the animal for hemoglobin production.

The practical significance of these findings is very interesting. If certain of our foods are low in total iron and only a portion of the total iron is available, many diets must supply an insufficient amount of iron. Several recent papers [Sturgis, Isaacs, Goldhamer, Bethell & Farrar (9), Kracke & Garver (10), Minot (11)] have dealt with the classification of anemias and have given special attention to those due to iron deficiency. Davidson, Fullerton & Campbell (12) have concluded recently from their studies in Aberdeen that anemia was found present in 41 per cent of the infants under two years, 32 per cent of pre-school children, 2 per cent of school children, 16 per cent of adolescent women, and 45 per cent of adult women. It was absent in adult males except in association with organic disease. Orr (13) has reviewed the rôle of inorganic elements in nutritional anemia, and he refers to the work of Werdinius (14) who found that in districts in Sweden, where the diet consists mainly of milk and cereals, anemia is common in adults but less frequent in children below thirteen years; reference is also made to the results of Orr & Gilks (15) who found in East Africa that anemia in children among cereal eaters occurred to the extent of 39 per cent of the girls and 48 per cent of the boys; the incidence was less than a fourth of this in tribes with a high intake of meat, blood, and milk. Most workers seem agreed that almost any form of available iron is a valuable supplement in these hypochromic anemias, especially in infants. Elvehjem, Siemers & Mendenhall (16) have found small amounts of iron together with copper (25 mg. iron as ferric pyrophosphate and 1 mg. copper as copper sulphate daily) very effective in increasing the hemoglobin content of anemic infants. Usher, MacDermot & Lozinski (17) obtained somewhat better results with iron and copper than with iron alone. The detrimental effect of excessive doses of iron has been pointed out by Deobald & Elvehjem (18).

The question of the necessity of other factors in addition to iron and copper for hemoglobin formation is still receiving attention. Robscheit-Robbins, Walden & Whipple (19) have shown recently that the activity of different liver preparations for the regeneration of hemoglobin in dogs after severe hemorrhage cannot be correlated with the total iron content of these preparations. A somewhat closer correlation might have been obtained if the available rather than the total iron content had been used. However, Sturgis & Farrar (20),

using Whipple's chronic hemorrhagic anemia method, found greater regeneration with liver than with an amount of inorganic iron equivalent to that in liver. They found that the greater response to liver was not "due to its content of amino acids which are present in casein." The additional factor or factors in liver may be concerned with the regeneration of constituents of the blood other than hemoglobin. In any case it is important to emphasize that if liver does supply additional factors for hemoglobin production they are present in milk because milk plus iron and copper allows optimum rates of hemoglobin formation. In this connection several workers have reported that the anemia produced in rats and children receiving goat's milk cannot be cured by iron and copper and that the anemia resembles the pernicious type. Alt (21) gives complete references to this work. However, Kohler, Elvehjem & Hart (22) have shown that the addition of iron and copper to anemic rats on goat's milk produced rapid hemoglobin regeneration although growth was much inferior to that generally obtained on cow's milk. Alt (21) also concluded that anemia from goat's milk showed maximal responses to iron and copper therapy.

Several new methods and modifications of older methods for the determination of total iron in biological materials have appeared (23 to 32). The bipyridine method described by Lintzel (25) will probably find most general use when applied to ashed samples. Since bipyridine unites with iron when combined with pyrophosphate there will be no inhibition due to the pyrophosphate formed during the ashing process as is the case when thiocyanate is used. Cooper (33) has used the bipyridine as well as the tripyridine method for the determination of iron in sea water. He found the amount of iron in true solution in sea water to be very small, less than 2 mg. per cubic meter. Tompsett (29), studying the use of thiolacetic acid in iron determinations, has shown that ferric iron forms complexes with certain biological materials. It is liberated by reducing substances such as thiolacetic acid and sodium hydrosulfite and by sodium pyrophosphate. Ferrous iron does not appear to form such complexes. This is interesting in connection with Lintzel's (34) conclusion that iron is absorbed from the intestinal tract in the ferrous state. Kletzien (35) has reported that the calcium content of the diet affects assimilation and utilization of iron.

Sachs, Levine & Fabian (36) studied the total iron content of human blood and factors affecting the changes in iron content. The

total iron content of human blood plasma has been found to be $1.25 \mu\text{g.}^2$ per cc. for males and slightly higher in the serum (23). Barkan (37) found 0.84 to $1.68 \mu\text{g.}$ of inorganic iron per cc. of serum and $17 \mu\text{g.}$ for whole blood. Tompsett (29) also found the corpuscles to contain a much greater amount of inorganic iron than the plasma and that at least part of it is in the ferrous state.

Marlow & Taylor (26) found 0.03 to 0.8 mg. of iron per day in the urine of normal men. Tompsett (29) found less than 0.01 mg. of iron per liter in normal human urine. McFarlane (39) has studied the distribution of iron in various animal tissues and the effects of proteolysis and autoprotoleolysis on the iron-containing components.

Several investigations concerning the daily total iron and copper requirement of humans have been reported. Daniels & Wright (40) conclude that 0.6 mg. of iron and 0.1 mg. of copper per kg. of body weight is sufficient for the maintenance and growth needs of normal children four to six years of age. Ascham (41) agrees with these figures. Farrar & Goldhamer (42) conclude that 5 mg. of iron is sufficient for normal adult males. Macy & Hunscher (43) as well as Coons & Coons (44) suggest that it is well to supply approximately 20 mg. of iron daily during pregnancy. Chou & Adolph (45) conclude that the copper requirement of man is about 2 mg. per day and that the adult human body contains 100 to 150 mg. of copper.

MANGANESE

Only a few additional papers on manganese in nutrition have appeared since the earlier ones [reviewed by Smith (46)] were published. Von Oettingen (47) has reviewed the distribution, pharmacology, and health hazards of manganese. Daniels & Everson (48) have studied the cause of the high mortality in the young born from rats on low-manganese diets. Young born from manganese-low mothers contained 65 per cent less manganese than those from mothers receiving adequate manganese and were not strong enough to survive birth, or if they did survive they were not active enough to suckle. However, mothers on the manganese-low diets were able to suckle young taken from mothers on a normal diet. Thus, manganese appears to play a more important rôle in the development of the fetus during gestation than in normal lactation after parturition as

² $1 \mu\text{g.} = 1 \text{ microgram} = 0.001 \text{ milligram} = 1 \text{ gamma.}$

was suggested by Orent & McCollum (49). Daniels & Everson found a considerable increase in the manganese store of young rats during suckling but very little increase after weaning when they were fed cow's milk. This suggests that rat's milk is higher in manganese than cow's milk. Ramage, Sheldon & Sheldon (50), who worked with human tissues, found the manganese concentration in fetal liver to show a definite increase during the last three months of fetal life, and that the amount of manganese in the liver decreased during the nursing period but increased again after weaning.

It should be pointed out that it is possible to reduce the manganese content of the diet for rats to such an extent that ovulation fails to occur and reproduction is therefore impossible. Orent & McCollum (51) have been unable to demonstrate the need of manganese either for growth or reproduction under the conditions of their experiments. This may depend to some extent upon the store of manganese in the rats when started on the experiment, for Keil, Keil & Nelson (52) obtained reproduction in first generations of rats fed milk plus iron and copper, but the second generation failed to reproduce unless manganese was added.

Everson & Daniels (53) suggest that the diet of children should contain between 0.2 and 0.3 mg. of manganese per kg. of body weight.

ZINC

Evidence is accumulating to show that zinc is an essential element both in animal and plant nutrition. Bertrand & Benzon (54) were probably the first workers who attempted to raise experimental animals on diets very low in zinc. Although their ration was probably exceedingly deficient in vitamins, they found that the group of animals getting zinc lived 25 to 50 per cent longer than those on the zinc-low ration. In 1927, Hubbell & Mendel (55) concluded that zinc is not merely an accidental factor in the nutrition of the mouse. Newell & McCollum (56) reported that no consistent differences in growth could be detected in animals receiving a ration in which there was almost a complete absence of zinc and control animals on the same ration to which zinc had been added. They did find, however, the zinc content of the rat's body to be variable and somewhat dependent upon the zinc content of the diet; also that the young born to a female which was on a zinc-low diet had a greatly diminished zinc content as compared with young born to a female on a stock diet. They concluded

that zinc is probably not an essential nutritional factor in the growth of the rat. Todd, Elvehjem & Hart (57) found that the growth of young rats placed in monel-metal cages and fed a highly purified synthetic ration which contained only about 1.6 mg. of zinc per kilo, but contained all of the known vitamins and essential mineral elements plus 2 cc. of milk daily per animal, was markedly inferior both as to rate of growth and maximum weight attained when compared to those receiving zinc. The ration with added zinc did not contain a maximum of all essential dietary factors, for although fair growth was obtained it did not compare with the growth which has been obtained on mineralized milk. Bertrand & Bhattacharjee (58) have attempted to improve their synthetic zinc-low ration, but even so the controls lived only fifty-seven to seventy-four days. In a more recent paper, Stirn, Elvehjem & Hart (59) have further substantiated their former work, which demonstrated that zinc was an essential element in the nutrition of the rat, by increasing the growth-promoting properties of the basal ration through the addition of certain water-soluble factors from milk serum and at the same time reducing its zinc content. As a result of these improvements greater differences were obtained in the rate of growth of the animals on the zinc-low and zinc-containing rations. These workers again found that there was some interference with the development of a normal fur coat in the case of the animals on the zinc-low diet.

An interesting suggestion concerning the possible action of zinc came from the work of Scott (60) which showed that zinc was a constituent of insulin crystals even when prepared by different methods. Scott & Fisher (61) have prepared crystalline insulin containing either zinc, cadmium, or cobalt, and the ash content of each sample was proportional to the formula weight of the metal which it contained. These workers (62) have also shown that the quantity of insulin which can be extracted from bovine pancreas bears no simple relation to the amount of zinc contained in the gland. Thus, there is at present no definite relation between zinc and insulin activity. Maxwell (63) found that the presence of small amounts of zinc salts in hypophyseal extracts produced a marked augmentation in the ovarian weight increase in young female rats. He also found the augmentation with pregnancy-urine preparations to be increased when zinc salts were added to the hypophyseal synergistic component.

Koga (64) has reported interesting results on the distribution of zinc in the animal body. Most of the values were obtained by the use

of the polarigraphic method. The liver and pancreas of various species contain the largest amount of zinc. Very little is found in the lungs, brain, and testicles. The amounts in blood showed marked variations. He found 1.4 to 2.1 mg. per kg. of human milk and 3.4 to 3.6 for cow's milk. The zinc content of colostrum was three times higher than that of normal milk. Todd, Elvehjem & Hart (57) found 2.68 to 2.76 mg. of zinc per liter of cow's milk. Unpublished results from the reviewers' laboratory show that the zinc content of active mammary gland is twice as high as that of the inactive gland.

COBALT AND NICKEL

These elements, if present at all, are very low in amount in living tissues and have not received the attention which copper, manganese, and zinc have.

The presence of traces of cobalt in certain biological materials has been reported by McHargue (65), Fox & Ramage (66), and Dutoit & Zbinden (67). Stare & Elvehjem (68) state that if cobalt occurs in biological substances it does so in extremely small amounts. One hundred grams of milk contained less than 0.01 mg. of cobalt, if any. By spectrographic methods, Blumberg & Rask (69) report no cobalt in milk.

Rats reared on whole milk mineralized with iron, copper, and manganese contained less than 0.01 mg. of cobalt in their entire body, while rats receiving the above ration plus cobalt showed easily estimable quantities of this element. Pigs fed a diet of mineralized milk likewise showed less than 0.01 mg. of cobalt in any of their organs, including liver, pancreas, heart, spleen, ribs, and long bones. A color sufficient to indicate a trace of cobalt was never observed in the analysis of any of these tissues. Stare & Elvehjem also found that the presence of 0.04 to 0.05 mg. of cobalt in the entire body of a rat was sufficient to produce marked polycythemia.

It is impossible to state that normal animals do not contain cobalt and that this element is unnecessary for normal development, but it must be concluded that if this metal is essential it is active in extremely small amounts. Recently, Underwood & Filmer (70) cured enzootic marasmus in sheep with the elements in the "zinc group" from the "iron-free" extract of limonite. Traces of cobalt alone have also given positive results. They suggest that enzootic marasmus is due to a deficiency of cobalt in the food and that cobalt must be regarded as an essential element in animal nutrition.

Fisher & Scott (62) were unable to detect either nickel or cobalt in the pancreas of cattle of any age and interpret their data as meaning that possibly only traces of these metals are present and that the amount is beyond the sensitivity of the analytical procedure used. Bertrand & Macheboeuf (71) as well as Zbinden (72) had reported the occurrence of nickel and cobalt in dried ox and human pancreas. Fisher & Scott insist that their failure to detect nickel and cobalt in ox pancreas was not due to substances interfering with the analytical method. The question of method is always the determining factor in much of the work with these "trace" elements. Blumberg & Rask (69) could not detect nickel in cow's milk.

As to the essential nature of nickel for animal life, we can only repeat what was said about cobalt; namely, that it is impossible to state with finality that the element is or is not essential; suspended growth with impaired function or definite pathological symptoms must be established on either nickel- and cobalt-low diets or nickel- and cobalt-free diets before definite conclusions can be made.

SELENIUM

Recent work indicates that plants in certain localities contain selenium in amounts sufficient to produce profound toxic effects when fed to animals. Seleniferous vegetation has been shown to be chiefly responsible for disturbances in livestock which have been referred to locally for many years as "alkali disease" and "blind staggers." In general, the two terms refer to chronic and acute cases of poisoning, respectively [Draize & Beath (73)]. Byers (74) and Beath *et al.* (75) have traced the history of the investigations on the selenium problem. Thus far, most of the research has been done by the South Dakota and Wyoming Experiment Stations and the United States Department of Agriculture.

The results of an intensive survey of the affected areas are reported by Byers (74). In an area of about 500,000 acres in South Dakota and Nebraska, soils were found to contain from a trace to 40 p.p.m. of selenium. Any soil containing 0.5 p.p.m. or more of the element may be regarded as potentially dangerous.

The absorption of selenium by plants has been studied chiefly by Hurd-Karrer (76). Various plants show widely differing abilities to take up selenium from a given soil. Those plants which normally absorb large quantities of sulfur were found to contain large amounts of selenium. The addition of elemental sulfur or gypsum to a sele-

niferous soil decreases the damage to the plant as well as the amount of selenium absorbed.

Although selenium forms chemical compounds which are strictly analogous to those of sulfur (77) it produces physiological effects which are similar to those produced by arsenic (78). Franke & Potter (79) state that the symptoms observed in rats which are fed toxic grains are quite similar to those which follow the ingestion of normal wheat to which sodium selenite is added. In both cases a severe anemia develops in most rats when the selenium content of the diet is 20 to 30 p.p.m. In some instances the animals die with acute symptoms without developing anemia. Although extreme inanition is observed, Franke & Potter (80) have shown that the pathology is caused by the toxicant, while the inanition is due to a voluntary restriction of food intake. The liver seems to be one of the primary foci of attack and becomes atrophied, necrotic, and hemorrhagic in varying degrees (81). Potter & Franke (personal communication) have shown that experimental animals are able to differentiate between diets of varying toxicity and that young animals are more susceptible than older animals. Byers (74) has made similar observations in the case of range animals.

A number of papers have appeared which describe the effects of the toxic foodstuffs upon domestic animals (73, 75, 82, 83). The most obvious symptoms are the loss of hair from the mane and tail of horses and from the switch of cattle and a general loss of hair from swine. Abnormal hoof development often occurs, and frequently there is a sloughing off of the old hoofs (83). Beath (75) has reported that these symptoms do not necessarily follow the ingestion of selenium-bearing plants. Draize & Beath (73) have described the microscopic and gross pathology of "blind staggers" and "alkali disease."

The acute cases of poisoning are almost invariably caused by certain plants which have been found to contain from several hundred to over a thousand p.p.m. of selenium (74). The minimum lethal dose of 2 to 4 mg. per pound of body weight, ingested orally (74), is in close agreement with the minimum lethal dose for selenium salts which are injected (78).

Much less selenium is required to produce cases of chronic poisoning. Byers (74) reports that toxic effects are produced by diets containing as low as 8.5 p.p.m. of selenium, while Franke & Potter (79), using young rats, produced death with diets containing 33.5

p.p.m. When the selenium content was raised to 52.1 p.p.m. the symptoms were virtually acute. Byers states that when selenium is present in the food it is found in all the body tissues as well as in the secretions and excretions. He suggests that selenium is, therefore, not permanently cumulative, although its effects may be permanent. He also reports the presence of selenium in milk and eggs from affected farms.

Franke & Tully (84) found that eggs from affected farms showed extremely low hatchability. About 75 per cent of the eggs which failed to hatch contained deformed chicks. Tully & Franke (85) found that the growth of chicks was inhibited, but no definite pathologic condition was produced by a diet containing toxic grain. Franke and coworkers (86) have also produced chick monsters by injecting extremely small amounts of inorganic selenium into normal incubating eggs.

Moxon & Franke (87) have shown that selenium salts inhibit the formation of carbon dioxide in fermenting mixtures, and Franke & Moxon (88) observed that toxic proteins lack the ability to stimulate yeast fermentation. Potter & Elvehjem (89) observed marked inhibition of the oxygen uptake of yeast cells on certain substrates in the presence of sodium selenite. Labes & Krebs (90) have demonstrated the inhibition of succinodehydrogenase by sodium selenite.

In growing plants the selenium is found in the aqueous extract (82, 91), but in grains such as wheat, the toxicant (92) and the selenium (93, 94) are found in the protein fraction and are not water-soluble. Franke & Painter have done considerable work on the chemical nature of the toxicant (93, 95, 96, 97). Their work indicates that the selenium is in organic combination in the protein molecule, probably in the form of compounds analogous to cystine and methionine. Selenium was removed from toxic protein hydrolysates by treatment with mercuric chloride, and the selenium-free amino acids were found to be non-toxic when fed to rats.

Knight (98) has discussed the relation of the selenium problem to the public health and states that "no great menace exists." Byers & Knight (91) and Strock (99) have recently reviewed some of the facts pertaining to the occurrence of selenium in soils and vegetation. Although selenium is almost certainly the chief causative factor of the pathology referred to above, other elements may also be involved. Vanadium has been found in toxic wheat, and vanadium, chromium, and arsenic in soils (100), while molybdenum and tellurium (75, 101)

have been found in native plants. In the words of Knight (98) "the development of the selenium problem gives added emphasis to the already recognized importance of trace elements in soils and soil products."

ALUMINUM, ARSENIC, BORON, AND SILICON

A number of other mineral elements could be discussed, but the experimental work is not extensive enough to allow definite conclusions. For a discussion of some of the less frequently studied inorganic elements found in living matter the reader is referred to the review by Sheldon (102) on the mineral basis of life. Of this group we will refer only to aluminum, arsenic, boron, and silicon.

Aluminum.—There is no new evidence to change the generally accepted conclusion that aluminum is a universal constituent of plants and animals. The human diet contains aluminum due both to the presence of aluminum in the foods and from cooking utensils. No knowledge is yet available to determine whether it is an essential element for the tissues or whether it is merely taken up passively. Cox, Dodds, Wigman & Murphy (103) and Deobald & Elvehjem (18) have found the addition of large amounts of aluminum salts to the diet to produce very severe rickets due to the retardation of phosphate assimilation. Bertrand & Serbescu (104) report that there is no evidence that ingestion of aluminum is favorable to the development of cancer.

Arsenic.—The question of whether arsenic is necessary for the proper functioning of living cells or whether its presence is merely due to ingestion of traces of this element in the food is still a debatable one. Guthmann & Grass (105) have reported results which indicate that arsenic may play some part in physiological processes. They found 63.8 $\mu\text{g.}$ per 100 cc. of blood in normal women. During menstruation the figure increases by 50 per cent, and in pregnancy it rises to a maximum during the fifth and sixth months, reaching 220 $\mu\text{g.}$ per 100 cc. Boos & Werby (106) claim that arsenic is not a normal constituent of the human body, but is present due to its ingestion with food. In addition, they state that the arsenic content of food is too large and must be viewed as a menace to the general well-being. Holmes & Remington (107) found a surprisingly large amount of arsenic in various samples of cod-liver oil. Recently Coulson, Remington & Lynch (108) made the interesting observation that the arsenic from shrimp was only meagerly stored in the bodies of experimental rats, while inorganic arsenic (As_2O_3), fed at the same

level, accumulated to a marked degree. According to these workers the arsenic in shrimp is in a complex combination which is made soluble and diffusible during digestion but which is rapidly eliminated by the kidney and cannot be liberated in the animal organism.

Boron.—Although boron has been shown to be essential for normal plant growth, there is no certain evidence of either its presence or function in the animal kingdom. Bertrand & Agulhon (109) claim to have demonstrated it in blood, and it has been found by spectrographic methods in milk by Papisch (110) and by Blumberg & Rask (69). Drea (111) found it present in food and water, but could not find it in any of the tissues of a chicken. He did detect it in egg yolk.

Silicon.—King, Stantial & Dolan (112) have reviewed some of the earlier papers on silicon. Kraut (113) reported an average of about 15 mg. of silica (SiO_2) per 100 cc. of human blood. Johlin (114) has demonstrated a combination of silicon and a carbohydrate in gelatin, ox blood, and human urine. Drea found silicon in milk ash (115) and in all tissue from normal chicks (111). Using a micro-method (116) for the determination of silica, King and coworkers (112) have found small but significant amounts in fetal tissue. They also found that in adult animals a considerable excretion of silicate occurs through the kidney and that the level of soluble silicates in the urine is influenced by diet. In experiments in which the urinary excretion of silica was markedly increased, there was no significant variation in the blood silica other than small temporary increases which indicate a very low renal threshold for silicates. King also found only one-tenth as much silica in the blood of normal humans as Kraut reported.

FLUORINE

The view has been held for a long time that fluorine is essential in animal metabolism—especially in the formation of the enamel of teeth. It is universally distributed in the tissues and is especially high in epidermal tissues and those tissues having a low metabolism. Administration of fluorides is accompanied by an unusual accumulation of this element in the bones and teeth, but other highly important organs such as the kidneys and thyroid gland show a markedly increased content of this element with increased fluorine intake (117). Sharpless & McCollum (118), working with rats, showed that this

species tolerates low levels of fluorine, and conclude that fluorine has no essential physiological function. Phillips, Hart & Bohstedt (119) used mineralized milk as the fluorine-low diet for rats. This diet contained 1 to 2 parts of fluorine per 10,000,000. Apparently normal teeth and normal animals were produced. Increasing the fluorine intake to 31 to 132 $\mu\text{g.}$ per day had no effect, stimulatory or otherwise. No one has as yet used a fluorine-free diet for animals. Until that is done a conclusive generalization that fluorine is not essential cannot be made. One can only say that on extremely low fluorine diets (0.1–0.2 mg. per liter of milk, equivalent to a daily intake of about 2 $\mu\text{g.}$) a graded increment of fluorine has not improved the growth or physiology of the rat.

Fluorine in too high a concentration in the diet can exert a distinctly toxic effect. What the toxic levels are can only be stated approximately. They vary with the duration of ingestion, the species, the kind of salts, as well as the concentration. Administering 2 to 3 mg. of fluorine as rock phosphate per kilo of live weight to cattle may show no bad effect in one to two years, but in the third year serious results will be experienced (120). Eight to 9 mg. of fluorine per kilo of live weight, given to the same species, results disastrously in a much shorter time. The symptoms shown are loss of appetite, a disturbed osseous metabolism which results in a great thickening and exostosis of the long bones and mandibles, and a flattening of the ribs. Microscopically various forms of degeneration including hyaline, hydropic, parenchymatous, and fatty degeneration were observed (120). These studies, supported by oxygen-uptake studies (121) on certain tissues, suggest that fluorine toxicosis produces its systemic reaction through an interference with cellular respiration and that the primary point of attack is the enzymatic systems of the body. Phillips, Stare & Elvehjem (121) state that there is a similarity between scurvy and fluorosis. They obtained evidence indicating that ascorbic acid deficiency and the deleterious effect of chronic fluorine toxicosis result primarily from disturbances in specific phases of cellular respiration. Phillips & Chang (122) also made the interesting observation that fluorine toxicity in rats causes the vitamin-C potency of the anterior lobe of the hypophysis and the suprarenal gland to increase.

In cattle the teeth are affected in chronic fluorosis with excessive abrasion of the second and third molars as well as premolars; hyperplasia is apparent in the enamel. Other farm animals, such as swine and chickens, are likewise subject to fluorine toxicosis, but the chick

is more tolerant of higher levels (123). Possibly fifteen to thirty-five times (70 mg. per kilo of body weight) the tolerance level for cattle can be borne by the chick without checking growth (unpublished data); they eliminate the element more completely. Sea water is relatively high in fluorine, and sea fish are high in this element: menhaden fish meal (dried) contained 0.74 gm. of fluorine per kilo; sardine fish meal 0.25 gm. per kilo. The reptilian character of the chicken may explain its higher tolerance for fluorine.

While increased fluorine intake does not lead to an increased fluorine output in cow's milk, in the case of the hen the fluorine content of the yolk of the egg is markedly increased. It appears that the fluorine wholly, or in large part at least, remains with the acetone-insoluble portion of the fat-like substances of the egg yolk. This suggests that fluorine is deposited in the egg in combination with the complex lipoids of the yolk. Apparently the hatchability of the egg is not impaired (124). Peters & Man (125) have recently reported the isolation of a lipid-chlorine compound from blood serum.

Recently attempts have been made to control human thyrotoxicosis by the use of fluorides (126). Goldemberg entertained the idea that fluorine toxicosis produced "cretinism," a condition he produced in sheep by poisoning with fluorine. He used sodium fluoride as the human therapeutic agent and reported that its administration, either orally or by injection, reduced the basal metabolism in the rabbit and the rat and lowered the basal metabolism in patients suffering from thyrotoxicosis, with relief of the accompanying symptoms. Gorlitzer (127) in 1932 reported the amelioration of symptoms of thyrotoxicosis and a return to normal metabolism by the use of warm water baths containing hydrofluoric acid. Raveno (128) has reported the effectiveness of sodium fluoride in reducing the basal metabolism of his patients where a regimen of alternate iodine and sodium-fluoride feeding was followed.

In laboratory work with normal rats (129) it has been shown that fluorine in the form of sodium fluoride does not lower the basal metabolism; that sodium fluoride augments and therefore enhances the toxicity of "hyperthyroidism" induced by feeding desiccated thyroid; that insofar as the spontaneous "hyperthyroidism" of toxic goiter and "hyperthyroidism" induced by the administration of desiccated thyroid are identical, to that extent sodium fluoride therapy is contraindicated; that a ration containing 0.15 per cent of sodium fluoride and 0.25 per cent of desiccated thyroid rapidly produces an

exhaustion of body weight and a fatal collapse, while either substance alone has no marked effect and is not fatal.

The fluorine menace of acid phosphates and rock phosphates (both used as sources of phosphorus in soil improvement) is discussed in a recent paper by Hart, Phillips & Bohstedt (130). They point out that plants grown on soils treated for sixteen to thirty-six years with rock phosphate and acid phosphate did not show consistent or greatly increased fluorine content over plant materials grown on plots receiving a low fluorine-carrying phosphate such as bone meal. On the other hand, drainage water from a single series of lysimeters in Chickamauga soil at Knoxville, Tennessee, showed a particularly high fluorine content when the soil had been treated with rock phosphate. The subject of fluorine in drinking water, as a danger to public health and cause of mottled teeth, is receiving constant attention. The minimum concentration of fluorine in water which will produce mottled enamel lies between 0.72 and 2 mg. per liter (131, 132, 133). Attempts are being made to remove fluorine from drinking water. Boruff (134) treats the water with aluminum sulphate, stirring thirty minutes, allowing to stand twenty-four hours, and filtering; 5 p.p.m. are reduced to 1 p.p.m. by this process. McKee & Johnston (135) remove the fluorine by adsorption on carbon. The water must have a pH of 3 or less at the time of treatment.

In addition, unpublished data from this laboratory indicate that fetal tissue and the tissues of young animals are much lower in fluorine than the tissues of mature animals. Similar observations were recorded by Sharpless & McCollum (118). The bearing of these data on the influence of food and water during the life of the animal in respect to its growth and physiological behavior is for the present a matter for speculation.

BROMINE

Since the findings of Zondek & Bier (136) that depressive psychotics show a low blood bromine, much interest has been aroused as to the rôle of bromine in animal life. Bernhardt & Ucko (137) claimed that the pituitary gland had a bromine content fifteen to thirty times that found in blood and that a bromine-containing compound stored in the pituitary circulated in the blood, causing sleep and a sedative effect. Because the above analytical data were obtained by the Pincussen & Roman method (138), which has been shown to be

untrustworthy (139, 140), the distribution and source of bromine in the animal body have been reinvestigated.

According to von Damiens & Blaignan (141) the bromine content of bread is 0.09 to 0.61 mg. per 100 gm. dry weight; potatoes 0.27 to 1.42; lentils 1.02; melon 9.45 to 26.2. Cooking salts contain small amounts of bromine; Dixon (142) reports 1 mg. per gm. of chlorine in a sample of salt examined. Dixon (142) determined the bromine content of the blood of persons with manic-depressive psychosis, untreated with bromides or iodides for at least six months previously, and found that the individual bloods examined showed the same type of variability in their blood bromine as do normal individuals. The bromine varied from 0.23 mg. per 100 cc. to 1.71 mg. Determinations on normal pituitaries and the pituitaries from idiots, maniacs, senile persons, etc., showed that their bromine content was of the same order as that of blood and that the data secured were inconsistent with the belief that a bromine-containing substance is either stored or secreted by the pituitary. Bromine and chlorine determinations on pigs' organs showed that the bromine/chlorine ratio was approximately that found in the ration fed. Dixon concludes that the rôle of bromine in the animal body is as mysterious as ever and that its presence is probably fortuitous and dependent upon food intake, and that "no evidence has been obtained in support of the theory that a bromine-containing hormone exists in the pituitary. Bromine metabolism does not appear to bear any relation to mental disease."

IODINE³

Progress in studies of the physiology of any element always hinges on the accuracy of the methods for its determination. Efforts are constantly being directed toward improvement in methods for the determination of extremely small quantities of iodine. McCullagh (143) describes such a method. The iodine is isolated for estimation by a process of alkaline digestion followed by acid distillation. The method estimates accurately as little as 1.05 μ g. of iodine as potassium iodide, 0.79 μ g. of iodine as *p*-iodobenzoic acid, and 0.71 μ g. of iodine in blood. If as accurate as claimed, it may displace the more complicated methods of von Fellenberg (144) and McClendon and associates (145), at least for certain materials. Trevorrow & Fashena

³ Cf. also this volume, p. 341. (EDITOR.)

(146) also outline a method for iodine determination in biological material. It is claimed that it will estimate accurately the iodine content of small samples (4 gm.) of material high in fats, such as butter.⁴

Studies continue to be made on the etiology of thyroid diseases as well as the comparative utilization of different forms of iodine. Elmer & Rychlik (147) studied the excretion of iodine in urine after the intravenous injection of 1.3 mg. in the form of inorganic iodine, thyroxin, and di-iodotyrosine. About 31 per cent of the iodine ingested as potassium iodide was excreted in twenty-four hours; 23 per cent appeared in six hours. After injection of pure crystalline thyroxin, thyroxin in alkaline solution, and di-iodotyrosine, the amounts of iodine excreted were 1 per cent, 7 per cent, and 9 per cent respectively in six hours, and 6, 14, and 32 per cent in twenty-four hours. If this work is confirmed, support would be given to the idea that organic forms of iodine would possibly be more economically used in the prevention of goiter than the common inorganic salts. The question of the cost of producing the organic iodine compounds is the only one involved because it is definitely established that inorganic iodine is as effective in the prevention of common goiter as are the organic forms. Elmer & Luczynski (148) working with rabbits studied the metabolism of iodine. They observed that after a meal the iodine in the bile was increased five-fold, whereas the level in the blood remained unaltered. They believe that the liver can hold back the iodine absorbed from the alimentary tract and return it by way of the bile, thereby establishing a cycle and preventing any large increase in blood iodine. Davis, Curtis & Cole (149) have studied the iodine content of human blood where no evidence of thyroid disease was apparent. In twenty-eight individuals they found values ranging from 8.5 to 16.2 $\mu\text{g.}$, with an average of 11.9 $\mu\text{g.}$ per 100 cc.; the average for women was 11.6 and for men 12.2 $\mu\text{g.}$ per 100 cc. This figure is in good agreement with data reported by Leipert (150), who found the iodine content of the blood of forty normal subjects to average 13 $\mu\text{g.}$ per 100 cc. It was hoped that the established blood norm could be used in clinical diagnosis of thyroid diseases. This hope has not been realized. Studies of the blood iodine in cases of non-toxic goiter, exophthalmic goiter, and myxedema by Elmer & Scheps (151) have

⁴ Kolnitz & Remington's modification of Karn's method is also of great promise. [*Ind. Eng. Chem., Anal. Ed.*, 5, 38 (1933)].

not proved useful since significant variations occur only in clinically obvious conditions. Nitschke (152) has made the statement that in rickets the blood iodine is low. This idea could not be confirmed by Toepfer (153) who studied the amount of iodine in the blood of fifteen rachitic children. Fasold (154) was likewise unable to confirm the idea of Nitschke that the thyroid is involved in rickets.

Spence (155) has written an extensive review concerning the etiology of goiter. From the literature cited he is of the opinion that goiter may be produced by an iodine deficiency in the diet, by the inability of the tissue to use the iodine adequately, or by the action of some unknown goitrogenic substance. Thyroid hyperplasia has been produced in rabbits by cabbage feeding (156). Thiocyanates or their precursors, the cyanides, which are common constituents of plants of the Brassica group, are held responsible for this goitrogenic action. Administration of various organic cyanides produces marked thyroid hyperplasia in rabbits, only slight hyperplasia in rats and mice, and none in guinea pigs or chicks. The goitrogenic action of the cyanides is believed to be due to the formation of hydrocyanic acid, which produces a compensatory activity on the part of the thyroid as a result of depression of tissue oxidation. Since the production of thyroid hyperplasia by cyanides needs such careful adjustment, there is some doubt as to whether the goitrogenic action of cabbage is really due to the action of cyanides.

Scheffer (157) thinks that simple goiter is not due to a deficiency of iodine in the ingesta and that lack of iodine in the environment where goiter is endemic is not necessarily the cause of the condition. Yet it must be stated that in regions where goiter is endemic the evidence (158) is convincing that the iodine content of water and food is low and the administration of iodine is followed by a correction of simple goiter. Scheffer found a negative iodine balance in exophthalmic goiter, while in simple goiter the iodine balance was normal. He holds that in exophthalmic goiter the thyroid and tissues are not able to assimilate the circulatory iodine.

McClendon (159) gives very definitely the amounts of iodine needed by children for the prevention of common goiter as suggested by Dr. Hans Eggenberger, Canton of Appenzell, Switzerland:

1. Iodine in very small quantity is a food.
2. One to 2 μg . daily per kilo of body weight.
3. If the average daily intake is under 1 μg . per kilo of body weight in any part of the world, the danger of goiter exists.

4. If the average daily intake is near 2 μ g. per kilo of body weight there is no danger of goiter, even though the susceptibility to goiter is increased by infectious diseases, high fat, or high cabbage diet.

Common salt continues to be the main vehicle for the distribution of iodine for human use and for farm animals. Goiter can easily be avoided with iodine in the salt in the proportion of 1 to 100,000 for daily use (158). In this country the amount of iodine in iodized salt is normally 0.015 per cent or 15 parts per 100,000. Recent recommendations to increase the amount to 0.15 per cent in the making of so-called "super-iodized" salt may not be in the proper direction as a public health measure. Recently, limited efforts have been made to supply iodine through the milk supply. Cows are fed iodized salt, iodized dry milk, or some other iodine carrier (160). The idea activating these efforts is to produce "a naturally combined organic form of iodine for human use." We are unable to find in the literature any sound evidence that organic iodine is any more effective in the prevention of simple goiter than is inorganic iodine. The claim of superiority of the "organic" mineral element over the inorganic dies hard. Marine (161) says that iodine is effective in goiter prevention when administered in any form and by any means. A number of workers have shown that the percentage of iodine secreted into the milk is low (15). Krauss & Monroe (162) state that 10 to 15 per cent of the iodine ingested appears in the milk; figures as low as 0.15 per cent have been reported (163). Apparently, supplying iodine in a goiter belt through the milk supply is not an economical process.

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DEPARTMENT OF AGRICULTURAL CHEMISTRY
UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

CLINICAL APPLICATIONS OF BIOCHEMISTRY*

BY JOHN P. PETERS, CLARENCE L. ROBBINS
AND PAUL H. LAVIETES

Yale University, New Haven, Connecticut

Throughout history the relation of clinical medicine to the more fundamental physiological sciences has been one of competitive collaboration. The strange experiments which nature provides in disease have been the starting point of much, if not most, of the investigations of physiology and physiological chemistry, even those which have ultimately led to the most esoteric fields; and in the course of these researches advances have come with a certain alternation from laboratory and clinic because, though planned experiments afford the advantages of voluntary control, the genius of man cannot yet compete with the capricious ingenuity of nature. This section should, therefore, properly emphasize more particularly the problems and contributions of medicine, than the beneficent influence of physiology on clinical practice. Since medicine has interests in the whole field of biochemistry it is impossible to make the initial reviews of this section comprehensive. Instead a few subjects have been chosen for discussion which will include the significant points in recent work and not only the studies of the last year. In the biochemical field the last decade has been signalized by rapid advances in knowledge of the functions of the endocrine glands and especially by the recovery (and sometimes even the identification) of the active principles of these organs. Although much of this physiological work received its initial impulse from medical problems, only a small part has ripened to the stage of clinical relevance. Nevertheless, to compress even this small fraction, with its implications, into the allotted space will tax the powers of the reviewer.

The description of Collip (1) in 1925 of the physiological action of a potent extract of the parathyroid glands, coinciding with Mandl's (2) report of the cure of *osteitis fibrosa* by removal of a parathyroid tumor, suddenly resolved a major physiological and clinical problem.¹ That parathyroidectomy was followed by hypocalcemia and tetany

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¹ Cf. also this volume, p. 342. (EDITOR.)

had long been recognized; but the calcium deficiency was generally ascribed to deficient absorption [see, for example, Salvesen (3)]. It has now been established beyond doubt that prepared extracts of the parathyroids and endogenous hyperactivity of the glands have similar general effects, causing: increased excretion of calcium and phosphorus, especially in the urine; decalcification of bones; hypercalcemia; and, with less regularity, diminution of serum inorganic phosphate, and metastatic calcification (see the following reviews 4, 5, 6, 7, 8, 9, 10, 11).

The most reliable reports (12) indicate that in human beings, at least, the activity of potent parathyroid extracts in subtoxic doses does not persist indefinitely, but after a certain time becomes exhausted. The refractory state which develops, though suggestively similar, is not quite comparable to the condition which Collip (13, 14) has demonstrated after administration of thyrotropic hormone. No evidence of inhibition of endogenous parathyroid activity can be detected in the serum or in the calcium balance. The difference in reaction to endogenous and exogenous hormone suggests the development of an immune reaction to the latter rather than a counter-hormone (5, 13, 14). This suggestion, while supported by no direct evidence, seems more consistent with clinical observations than the counter-hormone theory. The metabolic disturbance of hyperparathyroidism may continue for an indefinite period without the appearance of a refractory state. Nevertheless, after removal of the parathyroid tumor the patients may develop the same refractivity as normal subjects to exogenous parathyroid extract (15). If the allergic hypothesis is accepted it can only be inferred that the hormone in available extracts is contaminated or combined with some substance which gives it antigenic powers which the native product does not possess. In this case more highly purified products may be expected to have a durable effect.

Regardless of its origin, the transitory effect of parathyroid hormone has definite clinical and physiological implications. First of all, its therapeutic value is limited to acute conditions, chiefly to the emergency treatment of tetany; it appears to be of no value for permanent substitution therapy in states of idiopathic or postoperative hypoparathyroidism (16), although it may be a life-saver when such conditions develop precipitately. Secondly, it is obvious that conditions of chronic hyperparathyroidism can be only imperfectly reproduced by its use. This may explain distinctions between clinical

and experimental hyperparathyroidism which have been too little emphasized.

The pathology in all cases of hyperparathyroidism is closely related to the physiological effects produced in animals by overdosage with parathyroid hormone, variations being explicable on the basis of differences of severity, duration, and modifying factors such as diet (9, 18). In all, generalized osteoporosis, varying in degree, is found with or without the formation of cysts and giant-cell tumors. In those cases in which relief has been secured by operative removal of parathyroid tumors or hyperplastic parathyroid glands reformation of bone with repair of osteoporosis and absorption of giant-cell tumors has been observed; but cysts persist, apparently without progressing, suggesting that these may represent effects of permanent destruction and cicatrization (9, 18). Evidences of bone destruction or absorption are found in elevated serum calcium and in increased urinary excretion of calcium and phosphorus.

Numerous explanations have been offered for these changes. One of the most ingenious, advanced by Albright *et al.* (12, 19, 20, 21), refers the alterations of calcium balance to depression of the renal threshold for inorganic phosphate, on the ground that hypophosphatemia is frequently observed in *osteitis fibrosa* and is the first disturbance demonstrable after injections of parathyroid hormone (12, 19, 22, 23). This conception embodies certain assumptions that are not in tune with the most recent physiological theory: first, the existence of a renal threshold for inorganic phosphate (24, p. 280), and second, a general reciprocal relation between the concentrations of calcium and inorganic phosphate in serum (*vide infra*). Albright (18, 20) himself admits that if the inorganic phosphate of the serum becomes normal or elevated the essential disorders of the disease, decalcification of bones and wastage of calcium and phosphate, continue. Collip, Pugsley, Selye & Thomson (25) have shown that hypercalcemia can be induced by parathyroid extract after removal of the kidneys. In general, hypophosphatemia, to which Albright attaches so much significance, appears to be the least constant of the disturbances which have been considered characteristic of hyperparathyroidism (18, 26).

The hypothesis first advanced by Greenwald & Gross (27), that parathormone increases the capacity of the serum to carry calcium by promoting the formation of complex compounds, such as those which are produced with citrate, has proved equally untenable. In spite

of the claims of Benjamin & Hess (28), Brull (29), Klinke (30) and others, the existence of such compounds in high enough concentrations to account for the hypercalcemia is extremely doubtful [see review by Schmidt & Greenberg (11)]. The recent work of McLean, Barnes & Hastings (31, 32) indicates that that fraction of the calcium of serum in hyperparathyroidism which is not combined with protein is ionized.

There is a growing tendency, therefore, to ascribe the primary disorder to the biological processes of calcification in the bones and to conceive of the hypercalcemia and calcium wastage as results of this destructive process (8, 9, 11, 25, 33). Schmidt & Greenberg (11) admit that renal excretion of calcium and phosphorus may also be affected. That those aspects of hyperparathyroidism not directly referable to the skeletal disease can be attributed to the hypercalcemia is attested by early experiments of Collip (34) on the effects of overloading the body with calcium phosphate, and the mass of experimental work on hypervitaminosis-D (11, 35). Clinical evidence for the osseous or biological origin of the hypercalcemia and the calcium and phosphorus wastage in hyperparathyroidism is found in the frequency with which similar metabolic disorders have been reported in patients with destructive tumors of the bone. Such disorders have been reported in patients with metastatic tumors of various kinds (15, 26, 36), most frequently, perhaps, in general myelomatosis (15, 36, 37, 38, 39).

It has been suggested by Barr & Bulger and Bulger, Dixon & Barr (15, 38) that in these cases there is hyperactivity of the parathyroids of a "secondary" or "compensatory" nature. In two instances tumors or hyperplasia of the parathyroid glands have been reported (15, 38, 40). However, enough cases with hypercalcemia have now been proved to have normal parathyroids at necropsy to make it clear that anatomically demonstrable abnormalities of these glands, at any rate, are not prerequisite for the production of hypercalcemia or calcium wastage in the presence of tumors which cause rapid destruction of bone (26, 36, 37). It seems most probable that serum calcium and calcium balance reflect the speed and severity, rather than the nature of, the destructive process in the bones. An objection may be raised to this proposal on the ground that the hypercalcemia or the calcium wastage can be modified or even prevented in experimental (41) or spontaneous hyperparathyroidism (15, 21) by dietary measures. The theory does not, however, in any sense deny

that calcium metabolism is, like other vital processes, a complex of equilibrium reactions. It has been demonstrated quite as conclusively that a negative calcium balance can be diminished by similar measures in advanced myelomatosis (15, 36) and in a case of Paget's disease with a predominantly destructive process (36).

Paget's disease has also been ascribed to hyperparathyroidism (42) and in one case described by Albright, Aub & Bauer (18) lesions resembling both Paget's and *osteitis fibrosa* were found associated in a patient with a parathyroid tumor. The general consensus of opinion would, however, exclude Paget's disease from parathyroid disorders, leaving it with a variety of other dyscrasias of bone still among diseases of unknown etiology (6, 18, 43, 44, 45). In these, hypercalcemia is seldom found. There is less regularity about calcium balances, which depend more on the diet and the speed of decalcification. In Paget's disease, for example, most observers have reported normal calcium balances, some even positive balances (44, 46), but Robbins & Kydd (36) found rapid wastage in one case.

The condition known as basophilism [Cushing's (47) syndrome] deserves especial mention because it represents a syndrome apparently of endocrine origin which has not been reproduced experimentally and bears no clear relation to the known functions of the various glands with which it has been connected. Among the frequent manifestations of the disease is general decalcification of the spine, which is probably largely responsible for the characteristic pains and deformities. Whether the pituitary or the adrenal or both are incriminated, there is no physiological evidence that either has a direct influence on calcium metabolism unless it be through the so-called parathyrotropic hormone of the pituitary (48, 49). Although hyperplasia or adenomatous conditions of the parathyroids are among the glandular abnormalities reported in certain cases (17, 50, 51) they have been absent in others (52, 53). Moreover, hypercalcemia and active calcium wastage have not been found in those cases in which they were sought (50, 51). Solution of the cause of decalcification in basophilism must probably wait upon the discovery of the exact origin of the disease. Meanwhile its existence must give pause to those who would lightly grant complete dominance over calcium metabolism to the parathyroids.

In a certain proportion of cases of hyperparathyroidism, osteoporosis, although present, is a minor occurrence; abnormal calcification dominates the picture (50, 54). In most instances the calcium

deposits appear only in the urinary tract, either as calculi in the renal pelves or the ureters, or as diffuse infiltrations of the parenchyma of the kidneys. Such calcification may arise from somewhat different causes than the metastatic calcium deposits produced by toxic doses of parathyroid hormone, depending merely on the precipitation of calcium phosphate by reason of the high concentrations of calcium and phosphorus in the urine. Even in predominantly osteoporotic cases renal calculi and tubular calcification occur with great frequency, resulting ultimately in impairment of renal function. The absence of hypophosphatemia in certain cases is attributed by Albright, Aub & Bauer (18) entirely to insufficiency of the kidneys. More important, perhaps, are the implications of renal calcification upon the treatment of hyperparathyroidism. It has been demonstrated that calcium wastage in this condition can be prevented or diminished by the administration of large amounts of phosphate (21) and measures (38) that promote calcium retention in normal subjects. Such measures, as Albright, Aub & Bauer (18) have pointed out, will tend, however, to exaggerate calcification of the urinary tract. The appearance of the latter, in advance of serious osteoporosis, may represent the resultant of relatively slow destruction of bone and either high intake of calcium and phosphate or other features which cause the excretion of calcium and phosphate to exceed their solubility in the urine. Renal calcification has been seen in patients with myelomatosis (55, 56, 57). Hyperparathyroidism may take an acute form in which osteoporosis and more diffuse metastatic calcification develop with great rapidity, yielding a clinical picture comparable to that induced experimentally by massive doses of parathyroid extract (58, 59); cases of this kind, however, appear to be rare.

Of the characteristic metabolic disturbances of hyperparathyroidism no one is found with absolute consistency except some degree of osteoporosis, which is usually quite distinctive. The irregularity of hypophosphatemia has already been noted. Hypercalcemia is more constant, but can occur in other decalcifying conditions (*vide supra*). Its absence has been noted in certain cases and is ascribed by Albright to retention of phosphate by insufficient kidneys (18); but cases have been reported in which both phosphate and calcium of the serum were normal (26, 60), possibly because the disease had advanced so far that available supplies of calcium phosphate were exhausted, because there was a transient remission, or because the dietary provision of

these salts was inadequate. Determination of calcium and phosphorus balances is far too complicated to become a general diagnostic procedure; and, furthermore, balances may be altered by diet (21, 38). Ultimately, then, diagnosis can not rest entirely upon chemical or metabolic findings, although these are of the greatest value.

Although hyperparathyroidism is amenable to relief by operative removal of adenomata or hyperplastic glands, the immediate effect of operation is usually to precipitate acute hypocalcemia and tetany (15, 18, 26). This has been attributed to compensatory hypoactivity of the remaining parathyroid tissue. This explanation, however, seems hardly necessary. It is enough to suppose that with removal of the decalcifying stimulus calcium and phosphorus are rapidly withdrawn from the blood by the starved bones.

This hypocalcemia may persist for a considerable period. It may be promptly remedied by injections of parathyroid extract; but in this, as in other conditions of hypoparathyroidism, the extract, after a time, loses its potency (15). Therefore recourse must finally be had to other measures. The first place among such measures must be given to the administration of soluble calcium salts (3, 9, 15).

Concerning the effects of vitamin D in hypoparathyroidism there is the same controversy in the clinic as in the physiological laboratory. Claims are made that the vitamin is effective (61) and as flatly denied (62), with deductions concerning the interaction of vitamin D and the parathyroid hormone, ending in the old stone wall of accessory parathyroids. The physiological aspects of the argument have been too thoroughly reviewed (11, 33, 35, 63, 64) to warrant repetition with mere clinical coloring.

Clinical and physiological problems are also closely akin in respect to the general question of parathyroid deficiency. Hypoparathyroidism of major severity is sometimes seen after thyroidectomy, or appears spontaneously in certain subjects. Usually, after a time, hypocalcemia and its symptoms disappear if proper treatment is instituted early, giving the impression that adjustment for absence of the parathyroids may be effected through some other agency than the parathyroid hormone (3, 15). In a certain number of cases, however, active spasmophilia disappears, but hypocalcemia persists (16, 19, 62, 65, 66), so-called "latent tetany." In these patients tetany can usually be induced by moderate stimuli which cause hyperventilation. The occurrence of such cases has led to the conclusion that recovery in the first class is due to the hypertrophy of accessory

parathyroid tissue. In a sense the parathyroids may be considered as non-essential for life, since it is apparently possible by regulation of the calcium and phosphorus intake (and possibly the administration of vitamin D) to maintain human beings with parathyroid deficiency in health for indefinite periods (66).

The condition of chronic hypocalcemia, untreated or insufficiently treated "latent tetany," however, is not ultimately compatible with well-being. Even if patients with this condition escape the periodic attacks of tetany to which most of them are subject, a certain proportion, for reasons that are still mysterious, lose their vision by developing cataracts (16, 66).

As far as the bones are concerned hypoparathyroidism does not seem to give rise to excessive deposition of calcium, as one would expect in the opposite state from hyperparathyroidism, even if the subject is provided with more than the usual quantities of calcium and phosphorus to prevent tetany. At least definite overcalcification has not been demonstrated in patients with latent tetany nor have they been shown to absorb and retain for long periods excessive amounts of calcium and phosphate. The immediate effect of parathyroidectomy is indubitably to move calcium from the blood to the bones and to promote retention of phosphorus and calcium (16, 38, 67). This process, however, is apparently limited or self-terminative. Ellsworth (65) claims, to be sure, that low phosphorus diets have a peculiarly beneficial effect in parathyroid tetany and can be employed for long periods without fear of osteoporosis, which suggests a tendency to economy in the expenditure of bone-forming material which the normal subject does not possess.

With the discovery of the chemical nature of vitamin D and its provision in pure or highly concentrated form it became apparent that excessive doses of the hormone produced serious pathological lesions, resembling in many respects those of acute hyperparathyroidism: hypercalcemia, calcium and phosphorus wastage, necrosis of tissues, and extensive metastatic calcification. The spread between therapeutic and toxic doses is so great that it is highly doubtful whether hypervitaminosis-D can ever become a clinical problem (11, 35). The relation of vitamin D to the parathyroids is of clinical interest merely because of its implications in the treatment of parathyroid deficiency. There are, however, in addition to rickets and those cases of osteomalacia which seem to be due to exogenous vitamin-D deficiency or to lack of calcium or phosphorus in the diet (68, 69),

certain conditions in which the absorption² of calcium and phosphorus from the gut is deficient and decalcification of the bones results. In these conditions hypocalcemia is common and tetany frequently results. Outstanding examples are steatorrhea (16, 70, 71, 72) and certain cases of nephritis (73, 74, 75, 76); but similar pictures have been reported in patients with diabetes (77) and a variety of other pathologic conditions. In steatorrhea the large fecal excretion of calcium has been ascribed to the formation of insoluble calcium soaps and imperfect absorption of vitamin D; in the other disorders mentioned there is no satisfactory chemical explanation for the defective absorption. In all types or classes, it has been claimed by one observer or another that large doses of vitamin D improve absorption and thereby promote recalcification of the bones. Unless it is assumed that vitamin D is absorbed with difficulty, these results would support those who claim that this accessory food substance, under all circumstances, specifically furthers intestinal absorption of calcium and phosphorus in contradistinction to those who believe that it has such an effect only on animals which are suffering from vitamin deficiency.

The last two years has seen some resolution of the long controversy concerning the state of calcium in the blood and its equilibrium with bone.³ The early stages of the controversy were reviewed by Peters & Van Slyke (7). Schmidt & Greenberg's (11) review has brought the subject more nearly to date. A simple physico-chemical equilibrium between calcium phosphate in serum and in bone, built on the solubility-product theory, promised much for the solution of problems of ossification and offered at the same time an explanation for the tendency for calcium and inorganic phosphate of serum to vary reciprocally. The capriciousness of this reciprocal relation, conclusive proof that calcium was deposited in bones and metastatic processes not as the phosphates of calcium and of carbonate, but as a complex salt of the apatite series, and the demonstration by Robison, Kay and others of the probable rôle of phosphatase in bone formation, proved the futility of this simple solubility concept. Unsatis-

² "Absorption" is used merely to express the difference between the amount ingested and the amount excreted in the feces with recognition of the fact that "diminished excretion by the bowel" might express the same concept as correctly or more correctly.

³ Cf. also this volume, p. 195. (EDITOR.)

factory estimations of ionized calcium, adsorption phenomena and disagreements in the measurement of diffusible calcium maintained the idea that there was in serum a fraction of calcium, non-ionized, existing as complex salts similar to those formed by calcium with citrate, although no appreciable amounts of such compounds could be demonstrated directly. Meanwhile, the fact that a large proportion of the calcium of serum was combined in an undiffusible, presumably non-ionized form had been generally accepted and prediction formulae for the estimation of this fraction had been devised on a purely empirical basis. McLean & Hastings (78) have recently shown, chiefly by means of a biological method (the frog heart) first proposed by Straub (79) for the measurement of ionized calcium, that calcium of serum behaves as if there were an equilibrium between calcium salts of protein and calcium and protein ions, defined by the mass law equation
$$\frac{[\text{Ca}]^{++} \times [\text{Protein}]^-}{[\text{Ca proteinate}]} = K$$
 in which K

varies with pH, but has the value 2.22 ± 0.07 at the pH of normal serum. The calcium of the undissociated salt, calcium proteinate, is not diffusible and is apparently biologically inert (that is, it plays no direct part in determining phenomena such as the disturbances of muscular irritability associated with tetany). From the equation it is evident that the concentration of the undissociated salt will vary directly with the total concentration in the serum of either calcium or protein. This explains the fact that in hypercalcemia both diffusible and non-diffusible calcium are increased (31, 32, 80). The fraction of the remaining diffusible calcium which is non-ionized in sera encountered in health or disease is almost negligible (32). This affords no explanation for the reciprocal relation of calcium to phosphorus. The regularity of this relation has, perhaps, been too much stressed. Certainly it is no invariable rule (81). If either phosphate or calcium is increased to an extreme degree a fraction of each apparently becomes non-filtrable and non-ionized (82, 83). From the quantitative relations between calcium and phosphorus both Greenberg (83) and McLean & Hastings (84) have concluded that this fraction probably consists of tricalcium phosphate in some colloidal form. Such compounds appear only when Ca^{++} or PO_4^- rises to extreme heights and therefore probably occur in serum only in the extreme hypercalcemia of acute hyperparathyroidism or the hyperphosphatemia of the most severe acute renal disease. Apparently they are rapidly and selectively removed from the circulation (84).

The work of McLean & Hastings emphasizes anew the importance of measuring protein as well as calcium in the serum to permit interpretation of abnormal calcium concentrations; their formula furnishes a means of estimating ionized calcium. This is peculiarly important in patients with hypoproteinemia of malnutrition or nephritis. In certain cases of myelomatosis high serum calcium is accompanied by hyperglobulinemia (36, 37, 55). The latter may be in itself an aid to the differentiation of this disease from hyperparathyroidism, in which the serum proteins are usually unaffected; it may also be responsible for the hypercalcemia (85). High calcium in these cases must then have a different origin from the accumulations without serum-protein abnormalities that occur in this disease and with other tumors of bone.

More than the usual concentrations of phosphatase⁴ (enzymes capable of hydrolyzing phosphate esters) have been discovered in the serum of patients with a great variety of disturbances of calcification, notably rickets, hyperparathyroidism, and Paget's disease (86, 87). This has been taken by Robison, Kay and others to support Robison's (88) thesis that the action of these enzymes on organic phosphate compounds in bone is the factor that controls the direction and intensity of calcification in bone. High serum phosphatase is found, apparently, in most diseases in which calcification is actively disordered, but is apparently unrelated to the causes or direction of these disorders (18, 26, 86, 87). Clinically, therefore, phosphatase determinations are as yet of little diagnostic value, although repeated measurements may serve as criteria of the activity or progress of diseases of bone.

The clinical signs and the metabolic and chemical disorders which characterize Addison's disease (subtotal or total destruction of the adrenal glands) have been carefully analyzed in the last few years and have been found to correspond closely to those which can be produced in animals by removal of the adrenal glands (89, 90, 91, 92, 93, 94). It is claimed that they are amenable to treatment with potent extracts of the adrenal cortex⁵ and there is unquestionable evidence that patients with Addison's disease are benefited by certain of these extracts (89, 92, 95). Whether such substitution therapy will be permanently successful remains to be seen. At present it is

⁴ Cf. also this volume, pp. 43, 197. (EDITOR.)

⁵ Cf. also this volume, pp. 213, 339. (EDITOR.)

impracticable because of the prohibitive cost and variable potency of available commercial products. Partly for this reason attention has been turned to symptomatic treatment of the metabolic disorders of the disease with the hope of alleviating symptoms and prolonging life. Since in Addison's disease there is usually a residuum of cortical tissue which sustains the patient unless an acute crisis is precipitated, this course promises to be not entirely unprofitable.

The most hopeful move in this direction thus far appears to be the sodium therapy of Loeb (90, 91, 92). The value of fluid administration, and especially salt solution, had been recognized by earlier observers; but Loeb first demonstrated that depletion of sodium followed removal of the adrenal glands from animals and occurred during the crises of Addison's disease. He also showed that the lives of adrenalectomized animals could be prolonged by administration of sodium salts and that patients could be brought out of the state of shock which characterizes crises and maintained for considerable periods if they were given enough salt (90, 91). These results have been verified by Harrop and associates (92, 96) and others (97, 98) in both animals and human beings. The fall of sodium in the serum, in which both chloride and bicarbonate share, is attended by a rise of non-protein nitrogen (chiefly the urea fraction), and later by an increase of potassium, inorganic phosphate and sulfate. The last seem to mark terminal renal failure, but the non-protein nitrogen rises somewhat earlier, while there is still an adequate flow of urine with reasonable preservation of the concentrating powers of the kidney (89, 92).

This general sequence of events is seen in other states of dehydration and shock. The association of hypochloremia, which involves reduction of sodium of serum, with nitrogen retention, has been observed in profuse vomiting or diarrhea and in advanced nephritis. Attempts have been made in all these conditions to ascribe the nitrogen retention to the hypochloremia, and such terms have arisen as "uremia from lack of salt" and "hypochloremic uremia." In most of these states, however, the loss of salt is referable to wastage through obvious channels, the nitrogen to oliguria or increased protein catabolism or both [see discussions in 7; 24, p. 289; 73]. On the other hand evidence that salt depletion in itself is deleterious is found in the rapid, favorable response to salt and the less favorable or even deleterious effects of water. The last two years has seen the accumulation of much direct and indirect evidence that sodium is completely

or almost completely confined to the extracellular fluids (99, 100). In this case it follows, as Darrow & Yannet (101) have demonstrated, that when the sodium concentration of the extracellular fluids is reduced the cells must imbibe fluid and swell in behalf of osmotic equilibrium. That this gives rise to serious symptoms resembling shock appears from their experiments on dogs and comparable experiments by Gilman (102) on rats. That similar shifts of water occur in patients under analogous circumstances is indicated by less direct experiments of Laviates *et al.* (99), based on comparative studies of serum electrolytes, electrolyte balances and measurements of water exchange. At the same time there is indisputable evidence that under certain dehydrating influences, e.g., calcium-chloride diuresis (103), potassium is swept out of the body, presumably from cells. What the circumstances may be which determine whether or to what extent osmotic compensation between cells and interstitial fluids shall be made by redistribution of fluid or by excretion of potassium remains entirely mysterious. In general it has been established that until dehydration becomes extreme the concentration of total base (electrolyte osmotic pressure) in interstitial fluids is given precedence over the volume of fluid in the body by the regulatory mechanisms which control these variables (24, p. 289). It follows that salt depletion usually entails dehydration. However, cases of nephritis have been observed [an example has been reported (104)] by the authors, who, when deprived of salt, developed extreme deficiencies of serum sodium without noticeable symptoms, with minimal dehydration, and with no significant change of blood non-protein nitrogen.

Loeb *et al.* (90, 91) have proposed and Harrop (92) has endorsed, as a diagnostic test for Addison's disease, the response of serum sodium to withdrawal of salt from the diet. In view of the cases just mentioned this test must be interpreted with caution in subjects with evident impairment of renal function. More important is the obvious inference that shock in the crises of Addison's disease cannot be attributed to sodium deficiency alone, but rather to some phenomena connected with this sodium deficiency. The relatively high water content and low salt content of the tissues (105) and measurements of electrolyte balances (106) in adrenalectomized animals, as well as the terminal hemoconcentration observed in both animals (107) and patients (108) suggest the conditions produced by Darrow & Yannet (101) and may be of some significance.

Britton & Silvette (109) have attached especial importance to changes in carbohydrate metabolism, especially deglycogenation of the liver and the tendency to hypoglycemia, which occur in adrenalectomized animals. Although hepatic deglycogenation seems to follow adrenalectomy regularly and spontaneous hypoglycemia is not an unusual event, the latter is not consistently found even in the acute crises of Addison's disease (92, 93, 95). Moreover it has been claimed that it is one of the disorders of Addison's disease that is not affected by cortical extracts (93). Long (110) has found that in depancreatized and adrenalectomized animals these extracts maintain life, but do not restore the function of the adrenal cortex that influences carbohydrate metabolism. This is part of the evidence which has led him to conclude that the adrenal cortex, as far as carbohydrate metabolism is concerned, plays only an intermediary rôle, under the influence of the adrenotropic hormone of the pituitary gland.

One great bar to progress thus far has been the apparently complete inactivity of cortical extracts when given to normal animals (89). It has been claimed that they have beneficial effects in various abnormal conditions (111, 112), but these claims have not yet been generally accepted. Evidence of positive cortical activity can as yet be found only in patients with tumors or hyperplasia of the suprarenal cortex. The syndrome which has been traditionally associated with such tumors consists of: hypertension, obesity of the head and trunk, changes of secondary sex characteristics and sexual function, frequently diminished carbohydrate tolerance, and, quite commonly, osteoporosis affecting especially the spine. This syndrome is indistinguishable from that which Cushing has attributed to overactivity of the basophilic components of the pituitary gland, as can be seen by comparison of Cushing's cases (47) with the series reported by Walters, Wilder & Kepler (113). The problem becomes only the more confused by the fact that in a large proportion of cases adenomata or hyperplasia of both adrenal cortex and pituitary basophiles have been found at necropsy (47), and by the claims that the symptoms of the disease are improved by either operative removal of an adrenal adenoma (113) or by Roentgen-irradiation of the pituitary gland (47).

The nature of the osteoporosis has already been discussed (*vide supra*). The disturbance of carbohydrate metabolism may vary from an intolerance to glucose, demonstrable only by excessive alimentary hyperglycemia, to a mild form of diabetes. To attribute this to the

adrenotropic hormone of the anterior pituitary would be a premature and precarious guess. In acromegaly, a disease which is associated with tumors of the eosinophilic cells of the anterior lobe of the pituitary, carbohydrate intolerance is also frequent (7). Nevertheless, there is no physiological evidence that the anterior lobe provides two hormones, both of which impair carbohydrate tolerance.

The experiments of Houssay & Biasotti (114), Lucke (115), Long (110), and others have given the *coup de grace* to the long untenable theory of the purely pancreatic origin of diabetes and its counterpart, hyperinsulinism. They have also removed the necessity of ascribing "pituitary diabetes" to the influence of the hypophysis upon the pancreas. There has been an inevitable swing in the opposite direction that has led to premature and misdirected efforts to cure all diabetics by attacking adrenals or pituitary. The moral of the downfall of the unitary pancreatogenous theory seems to have been lost in enthusiasm over the new discoveries. Criteria must be found by which diabetes of various origins may be distinguished before the newer physiological knowledge can be profitably exploited in the clinic.

Meanwhile, from an entirely different direction the placid traditions of clinical diabetes have received a shock. The importance of the diminished glucose tolerance which results from carbohydrate starvation, so ably demonstrated by Bang (116), has at last received general recognition. This has led to various modifications of the dietetic treatment of diabetes. It has also stimulated new lines of thought concerning the nature of clinical diabetes, of which an example is found in the work of Himsworth. From a detailed analysis of the successive changes in the concentrations of glucose in arterial and venous blood after the administration of glucose or insulin or both (117), together with a study of the effects of carbohydrate starvation on these phenomena (118), he has concluded that the action of insulin requires not only the presence of this hormone, but also its activation by some secondary process which is inhibited by carbohydrate starvation. Although his proposal that diabetes may originate in impairment of this activating mechanism is still more interesting than convincing, he has built a strong case against the theory that "starvation diabetes" is due merely to the inhibition of insulin secretion. Soskin *et al.* (119) have also challenged the idea that administration of carbohydrate accelerates production or delivery of insulin by the pancreas, by reproducing in the depancreatized

animal (maintained in a constant state by continuous intravenous injection of glucose and insulin), after ingestion of glucose, blood-sugar curves that are indistinguishable in form from alimentary glycemia curves of normal animals; even the postalimentary hypoglycemic reaction is reproduced.

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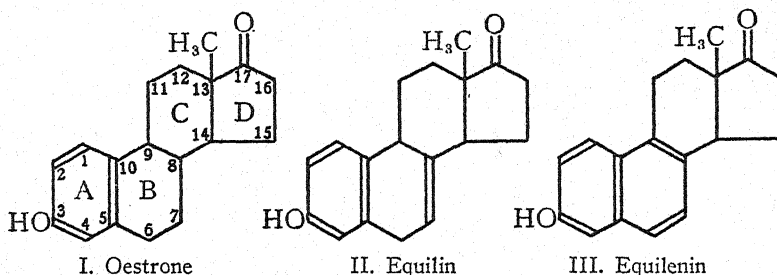
DEPARTMENT OF INTERNAL MEDICINE
SCHOOL OF MEDICINE, YALE UNIVERSITY
NEW HAVEN, CONNECTICUT

THE HORMONES*

By EDWARD A. DOISY AND D. W. MACCORQUODALE

St. Louis University School of Medicine, St. Louis, Missouri

*Ovary; oestrogenic compounds.*¹—At the time of the last report the remaining points of uncertainty concerning the structure (except for steric configuration) of the oestrogenic hormones: oestrone (I), equilin (II), and equilenin (III), were the position of the carbonyl oxygen in the pentatomic ring, the location of the quaternary methyl group and, in the case of equilin, the location of the ethylenic double bond. The experiments of Cohen, Cook & Hewett now serve to demonstrate the location of the carbonyl oxygen at position 17 and the quaternary methyl group at 13 in these three substances.



Dirscherl & Hanusch have dehydrogenated equilin by means of palladium and obtained a naphthalenic compound almost identical with equilenin, the slight differences probably being due to impurities difficult to eliminate. This indicates that the ethylenic double bond in equilin is in ring B. The ultraviolet absorption spectra of equilin and oestrone have been measured by Dirscherl & Hanusch and also by Cook & Roe, and found to be almost identical. The latter investigators point out that if the double bond of ring B were conjugated with the aromatic ring (position 6,7 or 8,9) there would be a wide divergence between the absorption curves of the two hormones. The unsaturation is therefore between carbon atoms 7 and 8.

The oestrogenic principle has been isolated from the liquor folliculi

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¹ In conformity with the practice of the *Annual Review*, the terminology advocated by a group of English investigators is used in the discussion of the oestrogenic compounds.

of hog ovaries and identified as dihydroxyoestratriene (dihydrotheelin) by MacCorquodale, Thayer & Doisy. It was separated from the active extract as the pure *m*-bromobenzoate (m.p. 154–155°) which upon hydrolysis yielded the hormone in the crystalline condition (m.p. 171–172°). The secondary alcohol formed by catalytic reduction of oestrone melts at 173° and its *m*-bromobenzoate at 156°. The melting point of the hormone crystals was not depressed when they were mixed with dihydroxyoestratriene. The oestrogenic activity of the two substances was found to be the same. David, deJongh & Laqueur have studied the biological properties of dihydroxyoestratriene and its esters.

This compound has also been isolated from the urine of pregnant mares by Wintersteiner, Schwenk & Whitman. These workers also isolated the stereoisomeric hydroxy phenol from the same source and found it to be identical with the δ -follicular hormone of Schwenk & Hildebrandt. From human pregnancy urine Marrian & Cohen have isolated a water-soluble complex which contains 55 per cent oestriol; it is apparently a monoglucuronide. Marlow & Groetsema have examined the ovary after removal of corpora lutea and liquor, and extracted a substance that causes vaginal introitus and uterine growth, the relative activities being such that it seems impossible for the reaction to be due to theelin.

Cartland, Meyer, Miller & Rutz have studied the properties of theelin prepared from stallion urine, human urine, and from theelol and have been unable to find any evidence for the existence of a β -form, thus confirming the work of deJongh, Kober & Laqueur and of Curtis, MacCorquodale, Thayer & Doisy.

Marrian & Beall have made a chemical investigation of the physiologically inactive alcohol, equol, which occurs irregularly in the urine of stallions and pregnant and non-pregnant mares and have concluded that it is an aromatic chromane or coumarane derivative.

1-keto-1,2,3,4-tetrahydrophenanthrol-7 has been prepared by Buteandt & Schramm who report that in a total dose of 9 mg. it gave no indication of physiological activity in castrated female mice. They also prepared 1-keto-1,2,3,4-tetrahydrophenanthrene and failed to obtain a response when 70 mg. were injected into mice. However, Cook, Dodds, Hewett & Lawson obtained 100 per cent response when 100 mg. of this latter compound were injected into thirty-six ovariectomized rats. Cook, Dodds & Greenwood also obtained plumage changes to the characteristic female phase by injecting this substance into

Brown Leghorn capons. Results obtained by Doisy, MacCorquodale & Thayer (unpublished data) are in agreement with the findings of the British workers, 100 per cent response being obtained upon injection of 25 mg. of 1-keto-1,2,3,4-tetrahydrophenanthrene into ten ovariectomized mice, the compound being administered in oil on three successive days.

Friedmann (1) has reported that fural pyruvic acid and benzal pyruvic acid possess oestrogenic activity of the same order as 1-keto-1,2,3,4-tetrahydrophenanthrene when injected into spayed mice. Cook & Dodds were unable to obtain oestrus in rats with doses of 100 mg. of these α -ketonic acids. According to Friedmann (2) 240 mg. are required to bring a rat into oestrus.

Earlier reports have indicated that the follicular fluid contains a substance other than the oestrogenic substance which stimulates metabolism. Khayyal & Scott report that liquor folliculi contains something other than the tested crystalline oestrogenic compounds which causes an increased oxygen consumption by the mouse uterus.

Continuing the study of the effect of injected oestrogenic substances on the ovary, Burdick & Pincus have studied the degeneration of ova in mated mice and rabbits. Ova were retained in the Fallopian tubes until degeneration occurred. Courrier & Gros (1) found that oestrin readily prevents implantation in the cat but that in the later stages of gestation pregnancy was interrupted with difficulty. Allen & Diddle, who have studied the effect of oestrin on the ovaries of immature monkeys, reported that immediately after cessation of a period of injections the ovaries were slightly decreased in size but that one month later the ovaries were again normal. Wade & Doisy carried the daily injections over a period of many months securing fecundity with both females and males. Litters were born in spite of the injections which, in view of experiments mentioned earlier (see Halpern & d'Amour), probably means accommodation of the genitals and breeding to an unfavorable stimulus.

The development of the corpora lutea of the rat in response to injections of oestrin [Hohlweg (1), Wolfe (1), Lane (1, 2), Selye, Collip & Thomson (1)] apparently falls in line with Hisaw's theory that the follicle secretion stimulates the production of the luteinizing hormone by the hypophysis.

Simpson & Burch, using small quantities of oestrogenic substance, produced a uterine reaction (glandular cystic hyperplasia) in the guinea pig without producing the response of the vagina character-

istic of oestrus, thereby indicating that, contrary to the mouse (Marrian & Parkes), the uterus is more readily stimulated than the vagina in this species. Using a chimpanzee and a sooty mangabey, Zuckerman & Morse obtained a "Swiss cheese" hyperplasia, which also occurs clinically, as the result of excessive secretion of the follicular hormone.

From the work on rabbits with uterine fistulae [Reynolds (1)] it is known that oestrin causes contractions of the uterus and that the active substance from the corpus luteum exerts an antagonistic effect. In studying the factors involved in labor, Robson (1) and Marrian & Newton have shown the existence of synergism between oestrin and the posterior pituitary principles in pregnant mice. The combination of the effects of quantities of each, which alone are insufficient to produce abortion, leads to a premature loss of the litter in a large percentage of experiments. Differing from the mouse, the rabbit, during most of the period of gestation, does not show a synergism between oestrin and pituitrin [Robson (2)]. During the late stages of pregnancy, after the luteal secretion has diminished, the synergism is evident. Robinson, Datnow & Jeffcoate were unable to produce abortion in the human with large doses of oestrin; in cases approaching full term, premature labor was induced in a few instances.

Prolonged daily injections of oestrin into rabbits leads in about one hundred days to a loss of motility [Reynolds (2)] and involution of the endometrium. However, the uterus still responds to progestin with the production of endometrial proliferation typical of the response to the corpus-luteum hormone.

The rôle of hormones in milk production is still a field of investigation. According to Asdell & Seidenstein, the ovarian hormones produce mammary growth in the hypophysectomized ovariectomized rabbit, thus indicating that the action is not through the hypophysis. Allen, Gardner & Diddle have found that ovariectomized monkeys treated with oestrin and subsequently injected with the lactogenic hormone secreted milk. Gardner, Gomez & Turner have shown that the duct system of the mammary gland of ovariectomized immature females and of male rabbits must be developed with an oestrogenic substance before galactin will cause the secretion of milk.

Both deJongh and Robson (3) have found that oestrin interferes with the production of milk. The latter injected large quantities of crystalline oestrone following parturition; the young failed to grow and died, but if the injections were stopped growth was resumed.

Burrows (1) found that prolonged injections produced hyperplasia and metaplasia of the prostate of mice [also see deJongh; Korenchevsky & Dennison (1)]; cessation of injections allowed restitution. In the male monkey oestrin produces growth of the fibromuscular tissue accompanied by a relative inhibition of the epithelial glands. Extensive stratification and cornification of the epithelium of the prostatic utricle occurred. It is probable that oestrin affects chiefly the structures of the male derived from the mesodermal tissue of the genital cord [Parkes & Zuckerman (1, 2), van Wagenen].

Another reaction in male mice, namely, the separation of the pubic bones, has been observed following a course of administration of oestrin [Burrows (2), Gardner].

Hain (1, 2) has made the interesting observation that the injection of the mother rat with crystalline oestrone during the later stages of pregnancy leads uniformly to the development of an abnormality in the urogenital region of the female offspring. Similar results are obtained if the mother is injected during the first postpartum days; the male offspring are not affected.

Some important data have been obtained on the effect of oestrogenic substances on the developing chick. Willier *et al.* (also Wolff & Ginglinger) have observed the development of an ovotestis from the left gonad with no effect on the right. In a series of thirty-six eggs Dantchakoff (1) obtained only females after injecting oestrin into the allantoic sac on the fourth day of incubation.

The identity of the stimulator of the bitterling's ovipositor with oestrin is still under discussion (Baumann & Szűsz, Fleischmann & Kann). On the basis of the relative activities of pregnant mares' urine and non-pregnant women's urine by the ovipositor and Allen-Doisy tests, Ehrhardt & Kühn conclude that the two substances are different but that they have some similar chemical and physical properties.

Metaplasia was produced in the rat uterus by massive dosage, cylindrical cells being changed to stratified epithelium (Selye, Thomson & Collip). Overholser & Allen produced atypical growths in the cervix of monkeys by using theelin and traumatizing the tissue. Engle & Smith observed a definite squamous cell development in cervical glands as the result of oestrin treatment but the changes had few of the characteristics of malignancy. Evidence of the carcinogenic effects of oestrin is still not conclusive (Campbell).

Further work on the relation of the hormones to menstruation in

the monkey leads to doubt concerning the causation of bleeding. The menstrual flow occurred in an hypophysectomized monkey after the administration of oestriol [Bachman, Collip & Selye (1)]. Engle and his coworkers have found that progestin administered following oestrin stimulation will prevent bleeding. Actually the bleeding begins four or five days following cessation of injections of progestin and is not inhibited by beginning the administration of oestrin at the time the injection of progestin is stopped. Hisaw's report that corporin will prevent post-castration bleeding is in line with Engle's work, and Zuckerman's (1) observations that the length of the menstrual cycle of sexually mature monkeys cannot be altered by injecting large quantities of oestrin during the latter part of the cycle also affords evidence against the theory of *oestrin privea* as the only cause of cyclic bleeding in normal primates. According to Corner, the first bleeding after starting oestrin administration comes at the expected time but the subsequent cycle is very much lengthened and bleeding occurs, although two to eight weeks later than expected, in spite of the continued injections.

The assay of the different oestrogenic compounds continues to be a source of difficulty and uncertainty. In 1932 the League of Nations set up a standard of oestrone which has been used by many investigators, but not always according to the recommendations of the League. Recently, Laqueur has shown that the monobenzoate of oestradiol gives very different values for the potency in international units when rats and mice are used for the comparison with the International Standard. If rats are used the value is much higher than that obtained with mice.

Another consideration of interest is the recognition (David & deJongh) that the vaginal reaction is only one of several that can be used to measure the biological activity and that a comparison of two different compounds may give very different potencies by different biological reactions. The response of the uterus, mammary glands, prostate, and seminal vesicles can be used but are less adapted to quantitative measurements than the vaginal smear reaction. However, we do not know which of these is the best indicator of therapeutic activity in the human.

The quantitative determination of oestrogenic substances in body fluids continues to be of interest. Cohen & Marrian (1, 2) have studied the conditions of hydrolysis necessary to secure a maximum yield of the free compounds and have applied a colorimetric pro-

cedure to the quantitative analysis. They have found that most of the oestrogenic compounds in the urine of pregnant women are in combination. With the advent of labor the oestrus-producing substances are excreted in the unconjugated form.

Smith & Smith (1) describe the details of the hydrolysis and determination by bio-assay using the vaginal reaction. Frank & Goldberger have improved the procedure for the assay of the oestrogenic substances of blood.

Data have been collected on: the excretion by pregnant mares in relation to the period of gestation (Kober); the concentration in the urine of the pregnant chimpanzee (Allen, Diddle & Elder); the nature of the oestrogenic substances in normal urine (Dorfman, Gallagher & Koch); the excretion in urine and feces during the menstrual cycle (Siebke); the amount present in the uterus and psoas muscle (Frank, Goldberger, Salmon & Friedman); and, the quantity excreted in the urine of patients having hydatid mole or chorionepithelioma [Smith & Smith (2)]. Allan & Dodds report a significant decrease in the excretion of oestrin following double ovariectomy of a pregnant woman.

Ovulation in the intact rabbit can be detected by the electrical changes accompanying the final enlargement and rupture of a follicle. The number of shifts in voltage is equal to the number of follicles which have ruptured (Burr, Hill & Allen). Marshall & Verney have induced ovulation in the rabbit by electrical stimulation.

Although clinical work is beyond the scope of this review it seems desirable to mention the favorable results of therapy in gonorrheal vaginitis of prepuberal girls. Several reports indicate agreement with the favorable results first obtained by Lewis. The report of Allen & Diddle indicates that any permanent damage to the ovaries is a remote possibility. It seems possible from the work of Papanicolaou & Shorr that the vaginal cycle may be used to obtain definite criteria of the activity of oestrogenic preparations used therapeutically.

Ovary; corpus luteum.—According to an agreement between prominent investigators of the progestational hormone, the pure chemical substance will henceforth be called progesterone (Allen, Butenandt, Corner & Slotka); the form melting at 128° is designated α and the one which melts at 121°, β .

Ehrhardt (1, 2, 3) reports the finding of as much as 10 rabbit units in one placenta (also Adler, deFremery & Tausk) but in a later paper states that frequently the placental extracts are inactive.

He also found small quantities in urine and in a mole. Elden determined the concentration of the luteal hormone in sows' corpora grouped in four stages of development; the greatest amount was found in corpora less than six days after their formation. From cows' corpora Kimura obtained 14 rabbit units per kilo, approximately one-third the quantity obtainable from sows. Human corpora contained less than sows', the test on 40 gm. yielding a negative result (Pratt).

Experiments with the two crystalline forms show that the inhibition of uterine motility is a property of the pure hormone (Allen & Reynolds). Experimenting with post-partum women, Falls, Lackner & Krohn found that progestin inhibited the oxytocic action of 1cc. of pituitrin. According to Robson (4) the effect of the oxytocic principle on the uteri of hypophysectomized rabbits is inhibited by progestin. The oestrus response of mice to oestrin can be suppressed with progestin (de Fremery, Kober & Tausk). This was confirmed by Allen & Meyer who showed that mucification identical with that of pregnancy occurs in the vagina. It is estimated that a weight of progestin at least 2000 times that of oestrin is required.

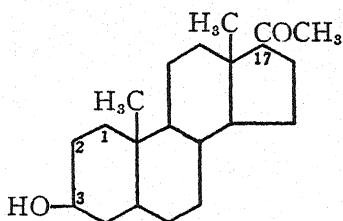
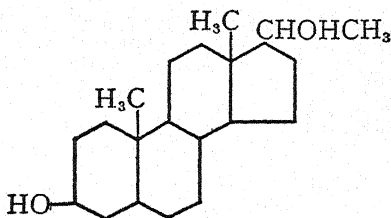
Clauberg & Breipohl found that injections of the luteal hormone would return the cell picture of castrated rats to normal but Hohlweg (2) using crystalline progesterone reported that castration cells persisted in spite of high dosage. On the other hand, injections of a lipid extract of corpora lutea produced pregnancy cells in the rat hypophysis (Charipper).

The importance of the corpora in pregnancy in the cat has been studied by Courier & Gros (2) who found that the loss of luteal function as late as the forty-fifth day of gestation (term, sixty-four days) interrupts the pregnancy. The loss of corpora in the rat leads to loss of the litter but Haterius found that if the ovaries and all fetuses except one were removed on the twelfth day (term, twenty-two days) but the placentas left intact, the animal carried the remaining fetus to term but parturition was not normal. He believed that the placenta supplied some progestin [see Selye, Collip & Thomson (2)].

Neuhaus has made a crystallographic and refractometric investigation of progesterone and has shown that the properties of the two crystalline modifications of the synthetic hormone are identical with those for the natural forms, luteosterone C and D.

The conversion of stigmasterol to 3-hydroxy-*alloetiocheryl-*

methyl-ketone has been accomplished by Fernholz. It proved to be identical with the physiologically inert *allo*-pregnanol-3-one-20 (IV) which accompanies the hormone in the corpus luteum. Butenandt & Mamoli (1) tried to convert this hydroxy ketone into progesterone by oxidizing it to the diketone, brominating this, and forming the unsaturated diketone by splitting out a molecule of hydrogen bromide. However, the bromination did not produce the 4-bromoderivative, substitution having taken place at some other point, probably carbon atom number two. The unsaturated diketone obtained was isomeric with progesterone and was inactive in 1 mg. doses in the Clauberg test.

IV. *Allo*-pregnanol-3-one-20V. *Allo*-pregnandiol

Butenandt & Mamoli (2) have found that when *allo*-pregnanolone (IV) is treated with alkali an equilibrium mixture is formed which contains an isomeric *allo*pregnanolone. Since the diketones obtained by oxidation of these isomers are different, epimerization of the hydroxyl group is not involved and it is suggested that the isomerization is due to a change in the steric arrangement of the hydrogen atom and acetyl group on carbon number 17.

Hartmann & Locher have isolated *allo*-pregnandiol (V) from pregnancy urine. Like pregnandiol it is probably formed by reduction of progesterone. *Allo*pregnanolone is probably an intermediate step in the reduction.

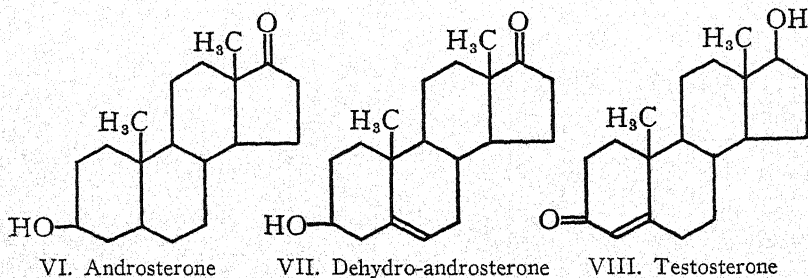
Slotta, Ruschig & Blanke have reported the preparation of a diketone (m.p. 188°) from progesterone and pregnenolone by catalytic reduction followed by oxidation. But on the basis of the accepted formulae for these two compounds only pregnandione (m.p. 123°) and *allo*-pregnandione (m.p. 200.5°) could be formed. Butenandt & Fleischer have repeated these experiments, and obtained from progesterone a mixture of pregnandione and *allo*-pregnandione, and

from pregnenolone only *allo*-pregnandione. In neither case was any compound melting at 188° obtained.

In the course of their investigations on the male sex hormone Ruzicka & Wettstein obtained androsten-3,17-dione (Δ^4 -etiocholen-3,17-dione) (IX) (see also Butenandt & Kudzus). Although this diketone is a lower homologue of progesterone, even 10 mg. does not produce the progestational reaction in the rabbit. Pregnenolone and pregnandione have also been found to be inert. These are further illustrations of the extraordinary specificity of the corpus luteum hormone as contrasted with the other reproductive hormones.

Male sex hormone.—Following the preparation of androsterone (VI) from *epidihydrocholesterol* by Ruzicka, Goldberg, Meyer, Brüngger & Eichenberger, the hormone has been prepared from stigmaterol and from sitosterol by analogous reactions, and from 3-hydroxy-*allo*cholic acid (Dalmer, v. Werder, Honigmann & Heyns) and from cinchol, the sterol of cinchona bark (Dirscherl).

Marker has contributed some valuable improvements to the method of preparation of androsterone from cholesterol. From extracts of male urine Butenandt & Dannenbaum have isolated a physiologically inactive unsaturated chloroketone, $C_{19}H_{27}OCl$, from which they obtained androsterone by reduction of the ethylenic linkage and treatment with potassium acetate followed by hydrolysis of the resulting ester. From this they concluded that the urine extracts contained an unsaturated derivative of androsterone which reacted with hydrochloric acid to form the chloroketone. Subsequently, a small amount of this dehydroandrosterone (VII) was isolated from the urine extracts. The same compound was prepared from the chloroketone by treatment with potassium benzoate followed by hydrolysis of the ester thus obtained. Later, Butenandt, Dannenbaum, Hanisch & Kudzus demonstrated that the chloroketone was in fact formed by the action of hydrochloric acid on dehydroandrosterone.



Dehydroandrosterone has been prepared from cholesterol by several groups of workers [Ruzicka & Wettstein; Wallis & Fernholz (1, 2); Butenandt, Dannenbaum, Hanisch & Kudzus; Schoeller, Serini & Gehrke], from stigmasterol by Butenandt, Dannenbaum, Hanisch & Kudzus and from sitosterol by Oppenauer. It gives an insoluble addition compound with digitonin (Schoeller, Serini & Gehrke) which shows that it does not belong to the *epi*-series, but that the steric configuration of the hydroxyl group is the same as that in cholesterol [Wallis & Fernholz (1)]. Since androsterone belongs to the *epi*-series and gives no precipitate with digitonin this difference affords a method for separating the two compounds which occur in about equal quantities in male urine. Dehydroandrosterone occurs in two modifications melting at 138° and 148°. Using the Tschopp method of assay the capon unit is found to be about 500 μg ., i.e., the compound is about one-seventh to one-tenth as active as androsterone.² By the Schoeller-Butenandt technique the unit is 600 μg . which is a potency of about one-third of that found for androsterone. There is no change in the potency when these compounds are converted to the acetates and injected as such, but the maximum effect is delayed. In stimulating growth of the genital tract in immature rats Butenandt, Dannenbaum, Hanisch & Kudzus find dehydroandrosterone to be about one-third as active as androsterone. For a significant effect the administration of 3 mg. per day for nine days is required. In the Allen-Doisy test it is inactive in 2 mg. doses. In their conversion of the chloroketone, $\text{C}_{19}\text{H}_{27}\text{OCl}$, into androsterone Butenandt & Dannenbaum found that in the step involving the treatment of $\text{C}_{19}\text{H}_{29}\text{OCl}$ with potassium acetate a small amount of an unsaturated ketone, $\text{C}_{19}\text{H}_{28}\text{O}$, was formed by the splitting out of hydrogen chloride. This unsaturated ketone was reduced to the saturated hydrocarbon androstane, $\text{C}_{19}\text{H}_{32}$. Since this was different from the isomeric aetiocholane from bile acids it indicated that androsterone does not belong to the steric series of cholanolic acid and pregnandiol but rather to the *allo*-series. Another compound obtained from the chloroketone was the ketone androstanone (aetio-*allo*-cholanone-17). This was also prepared by Fernholz & Chakravorty by the oxidation of cholestane. The bird unit is 2 to 3 mg. as compared to 0.15 to 0.2 mg. for androsterone.

Similar to the case of the female sex hormone, it has been shown [Ruzicka, Goldberg & Meyer (1); Butenandt & Tscherning] that

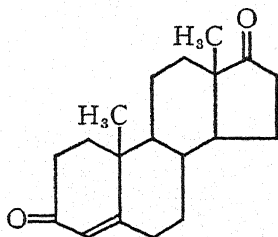
² 1 μg . = 1 microgram = 0.001 mg. = 1 γ .

reduction of the carbonyl group in androsterone to a secondary alcohol group results in a compound of considerably enhanced physiological activity. The androstandiol thus obtained has about three times the comb-stimulating potency of androsterone. No qualitative differences in action could be detected. On the prostate of the castrated rat the diol is more active per weight unit but is less active per capon unit than androsterone; on the seminal vesicles it is more active by both methods of comparison [Callow & Deanesly, Korenchevsky & Dennison (2)].

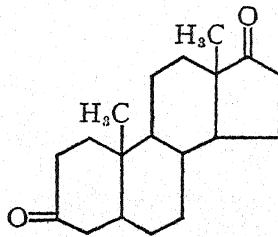
The physiological and chemical differences between hormone preparations from urine and testes have been abundantly demonstrated. In general the urine preparations are more active in promoting comb growth while those from the testicle have the greater effect in the rat test. Gallagher & Koch have shown that the active principle of the testes is destroyed by boiling alkali. This instability toward alkali is also a property of the $\alpha:\beta$ unsaturated ketone, progesterone, and on the basis of this Ruzicka & Wettstein suggested that the active principle in the testicular extracts is probably androstendione (IX), or Δ^4 -androstenol-17-one-3 (VIII). Almost simultaneously the same conclusion was reached by Wallis & Fernholz (3), and by Butenandt & Hanisch (1), and also by Tschopp who found androstendione is very active on the accessories of castrated rats. The isolation of the hormone from the testicle (testosterone) was reported by David, Dingemane, Freud & Laqueur who obtained it as a crystalline substance with a melting point of 154° and a potency of 10 $\mu\text{g.}$ per bird unit. According to these workers testicular extracts also contain a substance which acts as an activator for testosterone but which has no effect on the activity of androsterone. The preparation of Δ^4 -androstenol-17-one-3 (VIII) from dehydroandrosterone was shortly reported almost simultaneously by Ruzicka and Butenandt & Hanisch (1, 2). It proved to be identical with the testosterone of David, Dingemane, Freud & Laqueur. The double bond assumes the $\alpha:\beta$ position to the carbonyl group as in the case of progesterone. The activity was found to be 25 to 30 $\mu\text{g.}$ in the comb test [Butenandt & Hanisch (2)]. On vesicular tissue it is more than five times as effective as androstendione (IX) and more than ten times as effective as androsterone. Dehydroandrosterone and androstendione are inactive in castrated female rodents but in immature females they induce vaginal opening and oestrus. Butenandt & Hanisch (1) interpret this as an indication that they are converted to oestrin in the ovary. Testosterone is

also active in this test but androsterone and progesterone are inactive. Warren finds that even 10 mg. of crystalline androsterone fails to produce oestrus in the ovariectomized mouse.

According to Tschopp the active substance isolated from boar testes by Ogata & Hirano is probably androstandione (X).



IX. Androstendione



X. Androstandione

Ruzicka, Goldberg & Meyer (2) found the capon unit for the mono-succinic acid ester of androsterone to be 80 μ g. and for the corresponding ester of dehydroandrosterone, 60 μ g. However, aqueous solutions of both esters were much less active in the seminal vesicle reaction of castrated rats than the oil solutions of the respective alcohols (Korenchevsky, Dennison & Simpson).

The weights of the capon unit of some other laboratory products as given by Butenandt & Cobler; Butenandt & Hanisch (2); and Butenandt, Tscherning & Hanisch are isoandrosterone, 1.4 mg.; isoandrostandiol, 1 mg.; androstanol-17-one-3, 50 μ g.; Δ^5 -androstendiol-3,17, 1 to 1.3 mg.; and Δ^4 -androstendiol-3,17, no response with 350 μ g.

Concerning the claims of Frattini & Maino (1, 2) for priority in the isolation of the male sex hormone, Tscherning has pointed out a number of respects in which androsterone differs from the crystals obtained by the Italian workers. The latter have not yet reported analyses nor described derivatives but it now appears evident that their active substance is not identical with either androsterone or testosterone.

Zimmerman has described a color reaction for sex hormones which may be applicable in colorimetric determinations. The reagent is *m*-dinitrobenzene and apparently it reacts with compounds of this class containing the CH_2CO grouping. Thus oestrone, progesterone, androsterone, and pregnandione respond; pregnandiol does not.

Greenwood, Blyth & Callow have reported an extensive study of the factors which influence the assay of androsterone by the comb-growth method. They considered proportionality of dose to response, amount and nature of solvent, method of injection, age and weight of capons, period of assay, and repeated use of capons. Callow & Parkes effected complete restoration of the comb with daily doses of 2.5 to 5.0 mg. of androsterone. The comb of the Leghorn responds more readily than the comb of the Plymouth Rock; hens are less responsive than capons.

McCullagh & Walsh have prepared water-soluble extracts of the testes which cause atrophy of the secondary sex glands of normal male rats.

Sex inversion of the incubated hen's egg which was produced by the female sex hormone did not occur in response to administration of a male sex-hormone preparation [Dantchakoff (2)].

An extract of urine, very potent with respect to the comb test, administered to castrated dogs caused a marked decrease in urinary total nitrogen and urea. The energy exchange and fat metabolism of an obese castrate were increased, but the heat production of a thin castrate was not altered (Kochakian & Murlin).

Hypophysis; anterior lobe.—Braier & Morea have found that hypophysectomized rats on a complete diet excrete normal quantities of total nitrogen and creatinine but on a protein-free diet less total nitrogen and creatinine and lose weight less rapidly than normal rats. Other data indicate a specific effect of the growth hormone on protein anabolism (Schaffer & Lee). Injected rats showed smaller quantities of tissue peptides, free amino acids, and urea than the control animals; hypophysectomy led to an increase of tissue amino acids and urea. The experiments of Nilson, Palmer & Kennedy show that the growth hormone increases the efficiency of utilization of food which, however, is not dependent upon improved digestibility. Single injections into thyroparathyroidectomized dogs produced a prompt calorogenic response and a storage of nitrogen (Gaebler). The excretion of water was diminished but later diuresis occurred. The increased oxidation of fat was not prevented by moderate amounts of carbohydrate food but was inhibited by calories from sugar equal to twice the rise in metabolism. Though increased skeletal growth results from the activity of this hormone, increase in bone phosphatase does not occur (Wilkins, Calhoun, Pilcher & Regen).

Other effects produced by injection of the growth hormone are:

(a) an increase in the weight of the hypophysis of the male but little effect on the female rat (Rubinstein); (b) a prompt diminution in the concentration of glutathione in liver but, on continuing the administration, a return toward normal and an increase in the quantity present in muscle (Goss & Gregory); (c) an increase in the rate of hair growth of hypophysectomized rats (Snow & Whitehead); and (d) a greater weight of the offspring of injected mother rats (Watts).

Valso found that the anterior lobes of the blue whale and cattle contain approximately the same concentration of the growth hormone. The acetone-dried and powdered glands retain activity after storage of one year. Purification effected by adsorption on Norite, elution with phenol, and precipitation with a mixture of alcohol and ether gave a product weighing only 0.025 mg. per growth unit (Dingemans & Freud).

Experiments designed to elucidate the relationship of the anterior lobe to carbohydrate metabolism have not given concordant results.³ According to Cope & Marks the anterior lobe of rabbits contains a principle that renders the glycogen stores susceptible to the mobilizing action of adrenaline. Hypophysectomized rabbits are not able to maintain the normal level of blood sugar even though the amount of glycogen in the liver is normal. The injection of insulin causes an increase in the adrenaline content of blood but the sugar level continues to diminish; furthermore, the response to injected adrenaline is subnormal. Similarly, experiments by Fluch, Greiner & Loewi indicate that the livers of hypophysectomized frogs do not yield glucose to a perfusion fluid containing adrenaline as readily as livers of normal frogs. This might be interpreted in favor of an additional factor besides adrenaline necessary in the breakdown of liver glycogen to produce blood sugar.

The experiments of Cope & Marks and Holden & Thurston point to the non-participation of the thyroid in the regulation of blood sugar and in the effects of anterior lobe extracts on liver glycogen.

Long & Lukens have found that either adrenalectomy or hypophysectomy diminishes the glycosuria and prolongs the life of depancreatized cats. Anterior lobe extracts did not increase the glycosuria and ketonuria of the depancreatized cats which had been adrenalectomized but did in those which had been hypophysectomized.

According to Dawson & Milne damage to the cranial fossa and

³ Cf. also this volume, p. 216. (EDITOR.)

decerebration near the pituitary of the rabbit result in hyperglycemia. On the other hand, Chaikoff *et al.* found that a sham hypophysectomy of dogs produced an increased sensitivity to insulin. These experiments are not contradictory since besides the difference in species, the time of study after operation differed markedly.

Anselmino & Hoffmann (1) have obtained evidence of the existence of a pancreatropic substance which causes a prompt fall in blood sugar and of two substances which produce hyperglycemia. Commercial extracts of hypophysis which cause a prompt rise in blood sugar are said to owe this effect to a posterior lobe principle.

Data on the concentration of the thyrotropic substance in various classes of beef anterior lobes indicate the relative paucity of this hormone in the fetus (Bates, Riddle & Lahr). The presence of thyroid-stimulating substances in the mid-brain and cerebrospinal fluid has been reported by Schittenhelm & Eisler. Furthermore, Sturm & Schöning have found that ovaries are a more potent source than anterior lobes. The preparation was obtained in a fairly pure form. Active extracts were obtained from other tissues, notably the medulla of the adrenal.

As a result of their work on thyroidectomized rabbits and other animals with parenchymatous goiter, Marine, Rosen & Spark conclude that the acidophilic granules of the anterior lobe are true secretion granules related to the functional activity of the cells; these granules may contain the thyrotropic factor.

Crude extracts of the pituitary were separated into gonadotropic and thyrotropic fractions by the adsorption of the latter on precipitated benzoic acid [Greep (1)] while McQueen-Williams (1) has obtained evidence that the thyrotropic and inter-renal activities are due to different substances by showing that the assays of the two do not bear the same ratio in rat and beef pituitaries.

The relationship existing between the dosage and the microscopic appearance of the thyroid of young guinea pigs (Kippen & Loeb) is advocated by Heyl & Laqueur as the basis for the most accurate assay. Chemists who do not have histological facilities may use the decrease in iodine of the thyroid of guinea pigs for assay (McCullagh & Stimmel).

Commercial thyrotropic preparations administered to fasting rabbits (Rothschild & Staub) do not affect the level of blood cholesterol or acetone but increase the iodine number of the depot fat, and furthermore, alter the water, urea, and sodium chloride balance. On the

other hand, Pugsley found that in both rats and dogs this hormone caused a fall in serum cholesterol.

Schultze has found that three months after the ovariectomy of rats some are fat and others thin. The former have inactive thyroids and an increased proportion of basophils in the anterior lobe; the latter, active thyroids. The hyperactivity of the thyroid of ovariectomized guinea pigs is due to excessive stimulation by the pituitary which can be repressed by the administration of di-iodotyrosine (Loeser). The pituitaries of animals treated with di-iodotyrosine contain less of the thyrotropic hormone than untreated females. The degree of proliferation in the thyroid of the guinea pig is related to the oestrus cycle (Chouke, Friedman & Loeb).

The parathyrotropic hormone produces enlargement of the parathyroids in rats and rabbits and microscopic evidence of increased activity in cats, dogs, and guinea pigs. Anselmino, Herold & Hoffmann (1) conclude that rats are the most suitable for tests of the potency of parathyrotropic extracts.

Continuing their work Riddle and associates have found that prolactin produces a marked decrease in the weights of the ovaries and oviducts, in the size of the comb, and the space between the pubic bones of fowls. The broodiness which is induced is not caused by injections of follicle-stimulating hormone, thyrotropic factor, progesterone, or oestrin. Possibly a related observation is the suspension of oestrous cycles of non-parous mice for a period of twenty to twenty-five days (Dresel) following injections of the lactogenic hormone. No changes were observed in the mammary glands, but the examination of the ovaries was not discussed.

Evidence accumulates on the factors involved in the secretion of milk. A sufficient stimulation of duct development by oestrin (Gardner, Gomez & Turner; Allen, Gardner & Diddle), either supplied by the animal itself or by a course of injections, is necessary. The luteal hormone stimulates the development [Anselmino, Herold & Hoffmann (2)] of the alveoli but, according to Gardner *et al.*, galactin merely produces secretion of milk from a prepared gland and is not a participant in the induction of growth. In hypophysectomized-ovariectomized rabbits, the ovarian hormones produced growth of the mammary glands, thereby indicating that these hormones do not act through the pituitary (Asdell & Seidenstein). Jeffers has made an interesting cytological study of the mammary glands of rats. The cytoplasmic inclusions, chondriosomes, fat drops, and pseudo-yolk

spheres are indications of metabolic activity of secreting cells. Lactation can be secured in pseudopregnant rats through the stimulus of suckling.

According to Anselmino & Hoffmann (2), the properties of lactogenic preparations indicate that this hormone is a chemical entity. Owing to the production of milk entailing a phase of carbohydrate metabolism the effects of active lactogenic preparations on the blood sugar of monkeys and rabbits were studied; no change in the level was observed (Nelson, Turner & Overholser).

The lactogenic factor has been found in the urine of lactating women (Lyons & Page). In their paper, it is stated that the crop glands of squabs one month of age will react to only 1 μ g. injected intradermally over the crop gland, whereas intraperitoneally 100 μ g. is the minimum quantity which produces an effect. Bates & Riddle have published the details of the process used to extract and purify the lactogenic factor. At pH 8 little loss in potency occurs as the result of boiling an aqueous solution for one hour.

Perla's experiments deal chiefly with the nature of the changes in the reticular zone of the adrenal cortex which culminate in atrophy following hypophysectomy. Chronic adrenal insufficiency in rats seems to impair growth so that the administration of the cortical hormone is without effect. However, growth is resumed when the growth hormone of the pituitary is administered (Grollman & Firor).

Anselmino, Herold & Hoffmann (3) believe that their experiments indicate that a factor differing from all of the other recognized principles affects the medulla. Their adrenotropic preparation does not alter the cortex but, within a few hours after injection, causes vacuolization of the cells of the medulla and a loss of the chromaffin substance.

Further work on the erythrophore stimulant indicates that it differs from the cortico-tropic substance (Jores) and from vasopressin, oxytocin, and the anti-diuretic principle (Downes & Richards).

According to Anselmino, Effkemann & Hoffmann, sera from persons fed on a high-fat diet contain a substance which seems to be identical with extracts of the pituitary which affect fat metabolism. Administration to rats produces an increase in the amount of total fatty acids of liver and in the degree of unsaturation and an increase in the acetone of blood and urine (Sievert).

Rats injected daily with extracts of bovine glands no longer respond with an increase of acetone after three months. It is interesting that these animals do not produce acetone in response to starvation or phlorhizin (Black).

More detailed data are now available on the content of the gonad-stimulating factor in pituitaries of male and female rats of different ages. Confirming and extending earlier observations Clark (1, 2) and McQueen-Williams (2) have found that the hypophysis of the immature female rat contains more gonadotropic hormone than the hypophysis of the mature female or immature male. Hypophyses of prepuberal males contain very little but the amount gradually increases until it exceeds the quantity present in immature or adult females. The hypophyses of male and female rats gonadectomized at the age of one day showed very little difference in potency at the age of sixteen to eighteen days; the potency of the females was slightly greater than the normal but the activity of the males was double that of normal males. Gonadectomy at the age of seven to nine days gives somewhat different results, the hypophyses of the females being more active than the males (Stein). Using the rabbit-ovulation test instead of immature rats or mice Hill (1) found that the pituitaries of male cats and rats were more potent than pituitaries of females of the respective species; in rabbits and dogs the reverse relationship was observed. Gonadectomy of male and female rabbits and male cats diminished the potency of their pituitaries. In a further study Hill (2) found a marked decrease in the activity of the rabbit's pituitary twenty-four hours after ovulation, followed by a gradual increase to very high values at the end of two weeks of gestation. In pseudopregnancy the rise occurred more promptly. Similar studies on mares have been conducted by Hellbaum.

In an investigation of a recently developed strain of hairless rats Emery found that the hypophysis contains more gonadotropic hormone than is present in the albino strain; after castration this relationship is reversed. The hairless female gives birth to young but lactation does not occur. The pituitaries of immature pigeons contain little or no follicle-stimulating hormone (Riddle & Schooley). The anterior lobe of female *Rana pipiens* is more potent in producing ovulation than the gland from males (Rugh).

The successful transplantation of testes of new-born males to litter-mate sisters leads to the development of the male type of hypophysis as evidenced by follicular stimulation with constant

oestrus but a failure of luteinization (Pfeiffer). Though the hypophysis of the castrate male of parabiotically combined rats does not supply luteinizing hormone, it has been shown that the luteinizing hormone is present in the hypophysis of castrated male rats. Implantation leads to the production of corpora lutea (Evans, Simpson & Pencharz). Lipschütz (1) reports that the luteinizing activity of the hypophysis of the female guinea pig is less than that of the male.

The effect of the hypophyseal gonadotropic hormones has been analyzed by Lane (1) who has found that in the immature rat the follicle-stimulating hormone increases the number of follicles while the luteinizing hormone has no effect on the number but causes a marked increase in the percentage of vesicular follicles. Casida (1) suggests that the luteinizing hormone is necessary for ovulation but that excessive quantities lead to atretic follicles. The combined effects of follicle-stimulating hormone and luteinizing hormone are necessary for ovulation; augmentation of effects results from the combination (Fevold, Hisaw & Greep). In the normal rat, the follicle-stimulating hormone increases the development of follicles which secrete oestrin; this substance inhibits the production of follicle-stimulating hormone and causes the secretion of luteinizing hormone; luteinizing hormone causes ovulation and the development of the corpus luteum with its internal secretion (Hisaw, Fevold, Foster & Hellbaum). Hisaw proposes a similar explanation of the primate cycle.

The prepuberal pig's ovaries do not respond to gonadotropic preparations before the follicles reach the vesicle stage. After fifteen weeks of age, ovulation could be produced [Casida (2)]. Precocious oestrus was obtained in immature guinea pigs [Greep (2)]. Daily administration of an extract of sheep's hypophysis to rats six to twelve days of age did not produce ovarian changes and oestrus until the animals were twenty-six days old. In such an experiment with the anterior pituitary-like hormone thecal luteinization occurs. Selye, Collip & Thomson (3) conclude that the ovaries of very young rats are not sensitive to pituitary sex hormone.

Loeb and his collaborators have pointed out that pituitaries of different species produce different types of reaction in the immature female guinea pig. Thus the anterior lobes of cattle, sheep, and pig cause rapid atresia of follicles with the formation of some pseudo-corpora while the anterior lobes of man, rabbit, rat, and cat produce an intensified growth and maturation of follicles and two types of

luteinization, one giving large and the other small pseudo-corpora. By treating the pituitaries before implantation with certain reagents the effects were markedly altered; for example, cattle anterior lobes treated with 95 per cent alcohol lost their injurious action on follicles and produced an effect similar to that of rabbit pituitary.

Similar to earlier experiments on doves, Witschi & Keck find that the male and female English sparrow respond to pituitary hormones but not to extracts of pregnancy urine.

The study of the effects of hypophysectomy has been extended to include the cat and ferret [McPhail (1)] and fowl (Hill & Parkes). The operation in the ferret at mid-term (period of gestation, forty-two days) leads to abortion or resorption; on the thirty-fifth day, young are born a few days later but lactation is not successful. McPhail (2) has studied the operative details of hypophysectomy in the cat and given a report of the effects in the pregnant and non-pregnant female and male. During mid-pregnancy abortion occurs but in the later stages living litters are produced but secretion of milk does not follow.

Hill & Parkes conclude from their study of plumage changes in hypophysectomized Brown Leghorns that the function of the thyroid is dependent upon the activity of the anterior lobe. Replacement therapy by the injection of an extract of ox anterior lobe failed to arrest comb shrinkage, testis atrophy, and plumage changes.

The study of the cytology of the anterior lobes of both sexes of several species has been continued and the effects of experimental procedures such as castration, cryptorchidism, and the injection of gonadotropic preparations studied [Ellison & Wolfe, Franck, Nelson, Wolfe (2) and others].

The serum of pregnant mares contains two gonadotropic principles, one luteinizing, the other follicle-stimulating. The former is very similar to the pituitary luteinizer but the latter seems to be different from the pituitary follicle-stimulating hormone (Saunders & Cole). The prolan-like substance is present in the serum of non-pregnant mares (Cole & Saunders).

According to Engle & Hamburger the effect of pregnant mares' serum on the ovaries of pre-adolescent monkeys resembles the results of the injection of extracts of urine of castrate women, but differs from the effects of pregnancy-urine extracts. The ovaries show growth and proliferation of large and medium-sized follicles but no thecal enlargement or atresia of the ova; sexual skin changes are

produced. Meyer & Gustus have also observed the ovarian and sexual skin changes in the monkey; regression follows the prolonged injections. On the other hand, Evans & Simpson (1) find that the testis of the immature pigeon is relatively insensitive to mare hormone.

Superovulation, with the production of large ovaries containing normal corpora, has been obtained in young rats by the injection of extracts of urine from women in the menopause or after castration [Evans & Simpson (2)]. Fiessinger & Moricard; Frank, Salmon & Friedman; Oesterreicher; and Lipschütz (2) have also observed luteinization due to extracts of these urines. Augmentation of the effects of extracts of menopause urine is produced by both the hypophyseal synergist and an extract of pregnancy urine (Evans & Simpson).

The quantitative determination of the gonadotropic factors gives higher values by alcohol precipitation than by benzoic acid adsorption (Fiessinger & Moricard; Frank, Salmon & Friedman; Katzman, unpublished). Levin & Tyndale have reported the successful use of tannic acid in the precipitation of the gonadotropic substance of urine of castrates (also used by Hellbaum for pregnancy urine).

Saethre has reported new values for the gonadotropic factor of the urine of mature and old men; in 25 per cent of the latter the amount was considerably increased. Using the synergism procedure developed by Evans, Freed found a luteinizing substance in the urines of prepuberal children. In normal menstruating women, Frank & Salmon have demonstrated an increase in the follicle-stimulating and the presence of the luteinizing factors during the ninth to twelfth days of the cycle.

Of twenty patients suffering from cancer, of which only five were genital cancers, the urines of sixteen gave a reaction in rabbits similar to the effects of prolan A in that luteinization was negligible (Aron). x-ray irradiation increased the output of gonadotropic substance which was later followed by a decrease (Baudler). In two cases of acromegaly the assays of urine became negative after x-ray irradiation (Margitay-Becht). If the excretion is continued after the expulsion of an hydatid mole it is probable that the patient has a chorion-epithelioma (Brindeau, Hinglais & Hinglais). The high excretion which is said to be characteristic of hydatid mole and chorionepithelioma has been observed by Heim during normal pregnancy.

Quantitative analyses of placenta have been interpreted as offering evidence of the placental origin of the gonadotropic substances

secreted during pregnancy [Smith & Smith (3)]. Allan & Dodds have observed no alteration in the output of a patient who was ovariectomized during pregnancy.

Though the rhesus monkey does not excrete gonadotropic material in the urine during pregnancy, Zuckerman (2) has found that the chimpanzee, a primate more closely related to man, gives a positive Aschheim-Zondek reaction. No false positive tests were obtained but a few negative reactions occurred with the urine of animals known to be pregnant.

The identity of the gonadotropic principle of the urine of men having testicular neoplasms continues to be of interest. With exceedingly large doses of purified pregnancy prolan the effects in pigeons and immature rats were similar to the effects obtained from such urines [Evans & Simpson (3)]. Freed & Coppock have also found a similarity in the reactions of immature rats and guinea pigs to extracts of the urine from pregnant women and men with teratoma testis. Cross experiments with rabbits immunized with the gonadotropic substances from the two sources indicated their identity (Twombly & Ferguson).

The injection of female armadillos with extracts of pregnancy urine causes the production of cystic and luteinized follicles; in a few instances follicles ruptured and corpora lutea, easily distinguishable from the corpora of pregnancy, were formed (Hamlett). In the male lizard injections during the winter months produced changes in the testes including spermatogenesis and accessories which are characteristic of the breeding season (Evans, L. T.).

Based upon Engle's evidence that injections of anterior-pituitary-like hormones induced the descent of the testes in monkeys, several clinical reports have appeared on the improvement of hypogenitalism (Sexton) and cryptorchidism (de Aberle & Jenkins). Webster's success with patients having undescended testes has led him to advocate that the surgical treatment should be resorted to only after the injection of the hormone has failed.

Hypophysis; posterior lobe.—The oxytocic activity of extracts of the blood of pregnant cows and women is not associated with any particular stage of gestation; in fact, blood drawn during parturition seemed to be inactive (Bell & Robson). Anesthetized cats and dogs eliminate the oxytocic substance in the urine after intravenous administration (Larson).

Studying the extracts prepared by Stehle's new process Holman

& Ellsworth reached the conclusion that the hyperglycemic action in dogs is due to oxytocin.

As is well known the uterus is less responsive to the oxytocic substance during the activity of the corpus luteum. Junghans has found a failure to respond in glandular cystic hyperplasia, a condition in which the corpus luteum is absent and the uterus abnormally developed.

Gulland & Randall have studied the effects of oxidation and reduction of the oxytocic hormone; reduction causes a diminution of activity of 50 per cent but the activity is restored by oxidation. The hormone contains a redox system with E° equal to $+0.025V$ at pH 6; the results are not inconsistent with the behavior of a disulfide-sulfhydryl system. Freudenberg *et al.* believe that oxytocin resembles insulin in being a peptide with a redox system in which sulfur plays a rôle. Cystine, tyrosine, and histidine have been isolated from preparations having an activity of 300 international units. Stehle & Fraser have modified and improved Stehle's process for the extraction of the posterior lobe hormones; the melanophore activity of the pressor fraction is almost entirely eliminated. Freeman, Gulland & Randall have studied the adsorption, cataphoresis, and electrodialysis of the oxytocic hormone.

Experiments with tissue cultures of neural, intermediate, and anterior lobes of the mouse indicate that the intermediate lobe produces the melanophore hormone, the neural lobe the pressor and melanophore hormones, and the anterior lobe neither of these substances (Geiling & Lewis). Anderson & Haymaker have observed the production of the melanophore substance by tissue cultures of the pars intermedia cells of rat hypophysis.

The study of antihormones has led to the discovery that the sera of pregnant women (eight months) will prevent the rise in blood pressure of cats injected with the pressor principle (Schockaert & Lambillon).

The macrocytic anemia, produced by injecting rabbits with posterior lobe extracts, is accompanied by changes in the spleen and bone marrow (Dodds & Noble; Gilman & Goodman). This anemia is not due to loss of blood from the gastric ulcers. These ulcers which are generally located in the acid-producing part of the stomach are associated with a failure of the gland to secrete hydrochloric acid in response to histamine (Dodds, Hills, Noble & Williams).

Permanent polyuria in cats is produced by bilateral injury to the

supra-optico-hypophyseal system which results in atrophic changes in the supra-optic nuclei and posterior lobe. Unilateral damage does not cause polyuria but transient polyuria may be due to irritative lesions of the anterior lobe (Fisher, Ingram & Ranson). In the rat, Richter found that complete hypophysectomy gave only a temporary polyuria but that removal of the posterior lobe, leaving a portion of the anterior lobe, produced permanent diabetes insipidus.

*Adrenal gland; cortical hormone.*⁴—Later work by Kendall and his associates indicates that the crystalline substituted glyceric aldehyde, $C_{20}H_{30}O_5$, is not the only active principle in the adrenal cortex. This compound must be supplemented with salt to maintain the blood chloride and urea at normal levels. A second factor in cortical extracts produces the same effects as the addition of salt to the diet of the adrenalectomized dog under treatment with the crystalline hydroxy aldehyde.

Wintersteiner & Pfiffner have obtained several different crystalline preparations from cortical extracts, the activity of all being so low that none could be regarded as the hormone. A very active extract assaying 400 dog units per mg. has been obtained.

Further work by Loeb, Atchley & Stahl leads to a belief in the importance of sodium chloride in the maintenance of normal osmotic relations. In both adrenalectomized dogs and patients suffering from Addison's disease salt has proved efficacious in relieving muscular weakness and in restoring normal conditions. However, with complete loss of function of the cortex, salt alone did not suffice and it was necessary to administer the hormone. However, Harrop and his associates have gone a step farther in finding that adrenalectomized dogs may be maintained in an approximately normal condition for at least five months by the administration of sufficient quantities of sodium chloride and sodium bicarbonate. The salts are given in quantities which maintain approximately the normal levels of the blood. The gastric juice contains hydrochloric acid and anorexia with hypoglycemia does not develop. Allers has also been successful in maintaining adrenalectomized dogs with salt and sodium bicarbonate or citrate. At the end of one hundred and fifteen days blood sugar, potassium, sodium, chloride, bicarbonate, and urea were normal.

In the rat, earlier work (Rubin & Krick) showing that salt will prolong the life of adrenalectomized animals has been partially con-

⁴ Cf. also this volume, pp. 213, 305. (EDITOR.)

firmed (Gaunt, Tobin & Gaunt). However, Britton & Silvette have found that in the cat sodium and chloride may be reduced to levels lower than those occurring in operated animals without the appearance of symptoms of insufficiency. As further evidence for their theory of the importance of the derangement of carbohydrate metabolism in adrenal insufficiency, experiments with the adrenalectomized opossum show a fall in blood sugar, muscle- and liver-glycogen, but an increase of sodium and chloride in the blood, and a diminished excretion of sodium chloride in the urine. The behavior of sodium and chloride in the opossum and dog are quite different (Loeb). According to Harrop fasted dogs maintained on cortical extracts or salt may suffer from hypoglycemia which can be relieved with intravenous glucose but do not show adrenal insufficiency.

A comparison of the effects of trauma in normal and in cortin-treated adrenalectomized dogs indicates that the latter are more sensitive (Parkins, Taylor & Swingle). They suffer from a rapid hemoconcentration, with a shift of water and electrolytes from the blood, giving a picture of acute adrenal insufficiency.

Nicholson & Soffer believe that the cardiac arrhythmia observed in adrenalectomized dogs is due to the disturbance of the electrolyte concentration of the plasma, particularly the increase in potassium.

Grollman, Firor & Grollman have described the results of oral therapy with cortin adsorbed on charcoal. The adsorbate was as effective as the same amount of hormone by intraperitoneal injection and the adrenalectomized rats showed normal growth curves.

Insulin.—The increase in purine metabolism observed in dogs after the injection of insulin seems to be due to the hypoglycemia; dogs in which lowering of the blood sugar was prevented by the administration of glucose showed no increase in blood uric acid (Chaikoff & Larson). Luck & Richmond found that irradiated insulin causes a lowering of blood amino acids in normal but not in demedullated (adrenal) rabbits.

The addition of zinc salts lowers the activity of insulin in mice 60 per cent; in rabbits a delayed but prolonged action was observed (Scott & Fisher). On the other hand, Maxwell & Bischoff report that basic ferric chloride prolongs and accentuates the action of insulin in both rats and rabbits. Treatment of insulin with benzylcarbonyl chloride destroys the hypoglycemic activity presumably due to the combination with amino groups (Gaunt, Higgins & Wormall). Cuprous oxide and phenylmercuric hydroxide, both of which react

with sulfhydryl compounds, do not inactivate insulin (Schock, Jensen & Hellerman). Under certain conditions benzoquinone causes loss of activity due perhaps to combination with amino groups. Certain thiol compounds differ from cystine in that they do not produce inactivation.

Duodenal extracts digested by pepsin lowered the blood sugar of normal rabbits (Heller) and of two depancreatized dogs for two months; oral administration was effective (LaBarre & Ledrut). Laughton & Macallum find that extracts which lower the experimental hyperglycemia of rabbits may be prepared from duodenum, gastric mucosa, and liver. Furthermore, Macallum reports the presence of an insulin-inhibiting effect in some duodenal extracts.

Cope & Marks believe that the anterior lobe contributes something which renders the glycogen reserve more responsive to adrenaline. An increased resistance to insulin and a greater response to adrenaline are produced by extracts of the anterior lobe. An increased output of adrenaline in response to insulin hypoglycemia in hypophysectomized rabbits was demonstrated.

In a study of the neurologic symptoms in the human, Dameshek & Meyerson found that the uptake of glucose and oxygen by the brain as measured by the comparison of the arterial and internal jugular vein were decreased. The pulse and spinal fluid pressures were increased.

*Thyroid gland.*⁵—The synthesis of the ketonic acid corresponding to thyroxin has been carried out by Canzanelli, Guild & Harington. This new compound is less active than thyroxin in stimulating metabolism, the relative effects being 3:11.

Anselmino & Hoffmann (3) have confirmed their earlier report that the blood of pregnant women contains a substance that increases the carbon dioxide output and the blood acetone and produces a fall in liver glycogen of rats. Thiessen has found that the serum stimulates metabolism in thyroidectomized rats. The experiments of Eufinger & Gottlieb, based on the earlier work of Blum, indicate that during pregnancy the balance between the thyroid hormone and its antagonist is disturbed with the result that the effect of thyroxin in accelerating the metamorphosis of the tadpole is not inhibited by the blood from pregnant women. Magistris reported the production of a protective substance by rats in response to desiccated thyroid; after an interval, a second course of thyroid had less effect on liver glycogen.

⁵ Cf. also this volume, p. 286. (EDITOR.)

A dog treated with thyroxin produced the protective substance, water-soluble and protein-free, which enabled rats to store liver glycogen in spite of the administration of desiccated thyroid. An ultrafiltrate from an anterior lobe extract had the same protective effect.

Hyperthyroidism, produced by desiccated thyroid, brings about a prompt degeneration of the corpora lutea of pseudopregnant rabbits accompanied by changes in the uterus indicative of the absence of the luteal secretion (Engelhart). In male rats the feeding of thyroid did not impair reproduction but did increase the weights of testes, adrenals, and pituitaries, and diminished the weights of prostates and seminal vesicles. The anterior lobes showed an increase in basophilic cells and in gonadotropic activity (Cohen). Thyroidectomy in male rats resulted in smaller reproductive accessories and the production of fewer sperm (Smelzer). Although signet-cells were present in the anterior lobe, no change was observed in the content of gonadotropic hormone.

That the thyroid is involved in polyuria induced by injury to the pituitary stalk is indicated by experiments (Mahoney & Sheehan) in which it was shown that thyroidectomy abolishes the increased urinary volumes. The polyuria was restored by the administration of desiccated thyroid.

Though thyroxin added to tissue has little or no observable effect, the isolated tissues of rats which were suffering from experimental hyperthyroidism showed a definite increase in metabolism (McEachern; Markoff). Scott has made somewhat similar observations with alligator blood in which an increased consumption of glucose and formation of lactic acid were found.

Calorigenic activities of the following compounds decrease in the order given: thyroxin, N-acetyl thyroxin, adrenaline, di-iodothyronine, di-iodotyrosine, and thyronine (inactive) (Thompson *et al.*). According to Salter, Lerman & Means the response of myxedema patients is the same for *d*- and *l*-thyroxin.

*Parathyroid gland.*⁶—The finding at necropsy of an increase in the size of the parathyroids of patients suffering from chronic renal disease (Pappenheimer & Wilens) has led to an experimental study of this relationship. Using rats one kidney was removed and the other damaged; an increase of the size of the parathyroids with only rare mitoses resulted (Jarrett, Peters & Pappenheimer).

⁶ Cf. also this volume, p. 295. (EDITOR.)

The use of the perfused frog heart [McLean & Hastings (1, 2)] as an indicator of calcium-ion concentration has led to the conclusion that the chief factors affecting the calcium-ion concentration of serum are: (a) the amount of total calcium, and (b) the quantity of serum proteins. The parathyroid hormone influences the total calcium but the amount of ionic calcium depends on the concentration of serum protein (McLean, Barnes & Hastings).

Further chemical studies indicate (Tweedy *et al.*) that the inactivation of the parathyroid hormone by nitrous acid is due to oxidation rather than deaminization. Reducing agents do not affect the potency.

Pineal gland.—Engel (1, 2) has reported the preparation of a water-soluble extract of the pineal gland which produces vaginal cornification in ovariectomized rats. By implantation or by injection of aqueous alkaline extracts of glands, he observed an inhibition of the activity of gonadotropic extracts injected concomitantly. The production of cornified smears by pineal implants was confirmed by Saphir, but Hartog Jager & Heil obtained only negative results with pineal extracts. In the rabbit, the injection of pineal emulsion seemed to reinforce the action of pregnancy urine; the number and size of hemorrhagic follicles were increased (Vinals).

The intraperitoneal administration to successive generations of rats of preparations of the beef pineal gland made by extraction with hot dilute hydrochloric acid and subsequent fractionation produced astonishing results. The rate of growth was retarded but the rate of development and the onset of adolescence were hastened. In comparison with normal controls the ears and eyes opened, teeth erupted, testes descended, vagina opened, and fur appeared earlier in life (Rowntree, Clark, Steinberg, Hanson, Einhorn & Shannon).

Thymus gland.—The astonishing experiments on the effects of thymus extract on the growth of rats have been continued to the ninth generation. Thymectomy of parent rats has retarded the rate of growth of the young offspring (Rowntree, Clark, Steinberg, Hanson, Einhorn & Shannon).

According to Spreter thymectomy alone produces no change in growth or calcium metabolism but, combined with parathyroidectomy, tetany and tooth defects do not occur.

Anti-hormones.—The administration over prolonged periods leads to a loss of response to the following hormones: thyroid [Romeis, Blum, Anselmino & Hoffmann (4)], thyrotropic (Siebert & Smith, Loeb & Friedman, Collip, Selye, Thomson & Williamson), ketogenic

(Black, Collip & Thomson), gonadotropic (Zondek, Selye, Collip & Thomson, Fluhmann, Ehrlich, Meyer & Gustus) and growth (Collip). In certain instances an antiserum has been produced which prevented the response of a fresh animal to injections of the hormone [Bachman, Collip & Selye (2) and others].

Many experiments point to a loss of response to the thyrotropic hormone after its chronic administration. Collip & Anderson have prepared a very active antiserum by injection into a horse over a period of four months. The inhibitory substance did not withstand boiling for three minutes and even lost a considerable part of its potency in the refrigerator in two months. Administered with thyrotropic preparations, it prevented changes in the thyroid and in metabolism; with thyroxin the expected increase in metabolism occurred. By proper adjustment of the quantities of thyrotropic hormone and the antagonistic substance, hyperplastic changes could be induced in the thyroid without changes in basal metabolism. By using thyroidectomized sheep Eitel & Loeser have obtained evidence that the thyroid plays a rôle in the development of the inhibitory substance. In analyzing this problem, Max, Schmeckebier & Loeb state that several factors should be considered: viz., the antagonistic relation between the thyrotropic and thyroid hormones; the refractoriness of the thyroid, which develops upon continued administration of iodine, and the possibility of a foreign protein carrier being responsible for the neutralizing substance which is produced.

With respect to the growth hormone, Evans, Pencharz & Simpson found that hypophysectomized rats failed to respond to purified preparations after a short period of growth. This could have been interpreted as the production of a neutralizing substance but these investigators found that rats which had ceased to grow responded promptly to a less purified anterior lobe extract; i.e., the cessation of growth was due to the absence of some factor other than the growth hormone. On the other hand, Collip prepared an antiserum by injecting a horse with growth preparations. This antiserum inhibited the growth of hypophysectomized rats injected with purified preparations of the hormone; upon substituting normal serum, growth was resumed.

In the case of the gonadotropic preparations the serum produced by the injection of human pituitary extracts did not prevent the action of the gonadotropic substance obtained from sheep's pituitary (Fluhmann). The serum of monkeys injected with pregnant mares' serum will not neutralize the gonadotropic activity of sheep or human pitui-

taries or the urine of pregnant women (Meyer & Gustus). Selye, Collip & Thomson (4) found that the ovaries of rats receiving daily implants of rat pituitaries for sixty-eight days were subnormal in size but that they responded to injections of the anterior pituitary-like hormone. However, Martins and Witschi & Levine have observed that, after several months of union, excessive ovarian and uterine development in parabiotic rats, one of which was a castrate, still prevailed. This leads one to wonder whether the neutralizing factor is developed toward the natural hormone or is produced only in response to the products prepared in the laboratory.

In an earlier paper (Black, Collip & Thomson) it was shown that rats treated chronically with the ketogenic principle develop a resistance to it and that their serum contains an inhibitory principle. Black has extended these observations by investigating whether the inhibition also extended to the ketogenic substance produced in the rat's pituitary. Female rats injected for three months with the ketogenic factor failed to show the usual acetonuria produced by fasting combined with phlorhizin injections.

Efforts to prepare an antiserum for oestrin, using rats as experimental animals, were unsuccessful (D'Amour, Dumont & Gustavson).

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DEPARTMENT OF BIOLOGICAL CHEMISTRY
ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE
ST. LOUIS, MISSOURI

THE WATER-SOLUBLE VITAMINS*

BY HANS VON EULER

Biokemiska Institutet, Stockholm, Sweden

INTRODUCTION

Our knowledge of the water-soluble vitamins is confined to two classes: the vitamin-B group and vitamin C. Especially in the vitamin-B group very important chemical progress has been made in 1935: the constitution of vitamin B₂ has been established and verified by synthesis and, in essential respects, the constitution of B₁ has been determined. Concerning vitamin C, the structure of which has been known for two years, our knowledge of its biochemical and physiological rôles has advanced remarkably and exact methods for its assay have been developed.

Besides B and C other water-soluble vitamins seem to exist. Ray, György & Harris mention an unknown factor in the eye lens of rats which is not identical with the known components of group B, and which is said to be present in raw egg white. The factor H which György (3) associates with another antidermatitis factor, probably B₆, seems to be closely related to the B group, if not a component of it. The factor J, found in lemons and berries, and especially in black currants, was biologically characterized by Euler & Malmberg by its antipneumonic effect on guinea pigs. This factor often accompanies vitamin C but as no other relations between the two vitamins are known the name C₂ does not seem justified.

VITAMIN B₁¹

Distribution.—The B₁ content of a great number of foodstuffs has been determined by Baker & Wright. Elvehjem, Sherman & Arnold, in studies of the vitamin-B₁ content of animal tissues, found that commercial canning might destroy up to 80 per cent of the vitamin. Scheunert & Schieblich determined the B₁ content of beer. They found it present to a small degree in dark beer having a high extract content. Concerning the B₁ content of different plant materials see Goricca *et al.*, Spruyt & Donath, Morgan *et al.*, and Vacca; of human urine, see Helmer.

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¹ Cf. also this volume, p. 32. (EDITOR.)

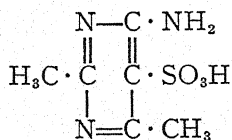
Assay of vitamin B₁.—The curative pigeon test with injection of the vitamin is not satisfactory when crystallized vitamin B₁ is used. (Tests with this method are reported by Ohdake & Yamagishi.) Kinnersley, O'Brien & Peters (1) consider the catatorulin test to be the most reliable method of assay. In this test Peters & Sinclair and Peters, Rydin & Thompson use the faculty of vitamin B₁ to restore a specific *in-vitro* respiration to avitaminotic brain. Poisons (fluoride, iodoacetate) reduce largely the catatorulin effect. By testing B₁ on the growth of rats Kinnersley, O'Brien & Peters (1) have established that 1.3 pigeon doses (1.1 international units) is sufficient for nearly maximum growth. The authors show by various tests that hydrochlorides of B₁ from different sources (Oxford, Göttingen, and Batavia) are identical. Schopfer (cf. also Schopfer & Jung) has proposed a new method of assay, using plant material (*Phycomyces*, *Rhizopus*, etc.). Concerning the assay of traces of vitamin B₁ with aseptic cultures of *Drosophila melanogaster* see van't Hoog.

Avitaminosis B₁.—In avitaminosis B₁ Thompson & Johnson, using pigeons and rats, found abnormally large amounts of bisulfite-binding substances in the blood, which probably consisted entirely of pyruvic acid. The accumulation of this acid is specifically related to avitaminosis B₁, because the blood of animals cured by B₁ had a normal concentration. It might be noted here that Lehmann found the occurrence of methylglyoxal in the urine of rats to be not specific for avitaminosis B₁. The activity of pancreatic lipase (rats) is reduced in B₁ deficiency (Sure, Kik & Buchanan). The chronaxie of the vestibular nerve is diminished in B₁ deficiency in pigeons (Mouriquand *et al.*). Gordonoff & Ludwig discovered the interesting fact that growth of fibroblasts from the embryonic heart or of cancer cells is checked in "beriberi plasma" (plasma from rats maintained upon a B-free diet).

Studies on clinical cases of B₁ deficiency are reported by Elsom, Maedr, and Ohomori *et al.* Therapy of human beriberi was successful with crystalline B₁ (Williams, Waterman & Keresztesy; Vorhaus, Williams & Waterman; Hermano & Eubanas).

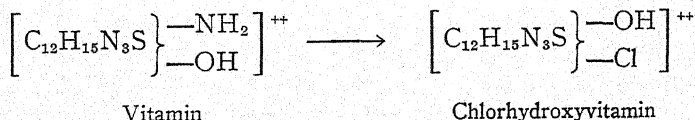
Chemistry.—Repeated analyses [Wintersteiner, Williams & Ruehle; Kinnersley, O'Brien & Peters (1)] have confirmed the formula first proposed by Windaus, Tschesche & Ruhkopf: C₁₂H₁₆ON₄S (2HCl for the hydrochloride). Kinnersley *et al.* (2) have reported upon improved yields of the crystalline vitamin from yeast (average 62.3 mg. pure hydrochloride from 50 kg. of yeast).

Moggridge & Ogston show by potentiometric titration the existence of two weak basic groups and one pseudo acid group in the B₁ molecule (cf. also Birch & Harris). After the detection of a thiazole nucleus in the vitamin, Williams and Ruehle found that one of the basic groups is of the same order of strength as nitrogen in 4-methyl-thiazole-ethiodide. The titrations gave evidence for the view that the thiazole nucleus exists in the vitamin in the form of a quaternary salt. Williams and his coworkers showed that vitamin B₁, on digestion with sodium sulfite at room temperature (pH adjusted to 4.8 to 5.0 with an excess of sulfurous acid), is quantitatively split into a sparingly soluble acidic product, C₆H₉O₃N₃S (I), and a chloroform-soluble basic product, C₆H₉ONS (II). The former has the properties and the absorption spectrum of a 6-aminopyrimidine. Strong hydrochloric acid converts it, with the loss of one mol NH₃, into a compound, C₆H₈O₄N₂S, having the properties of a 6-hydroxy pyrimidine. Sulfur is bound in these derivatives as a sulfonic acid group. Williams and his coworkers provisionally suggested for I the structure of an ethyl-amino-pyrimidine-sulfonic acid, but a paper by Windaus, Tschesche & Grewe (1) makes it appear most probable that I has the structure of a dimethyl-amino-pyrimidine-sulfonic acid:



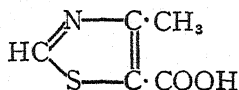
I

The vitamin itself is converted, by the action of concentrated hydrochloric acid, into a compound (C₁₂H₁₆ON₃SCl)⁺⁺Cl₂⁻ ("Chlorhydroxyvitamin"). The reaction is interpreted as follows:



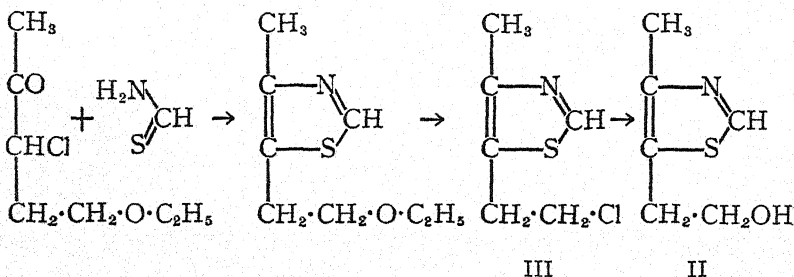
Product II, when treated with concentrated hydrochloric acid, gives a compound, C₆H₈NSCl(III) (Cl non-ionic); the absorption spectra of II and III are nearly identical. With methyl iodide a typical quater-

nary salt is obtained. Oxidation of II with nitric acid gives an acid, $C_4H_4NS \cdot COOH$, identical with the product obtained by Windaus, Tschesche & Grewe (2) by oxidation of the native vitamin and identical with 4-methyl-thiazole-5-carboxylic acid (IV):

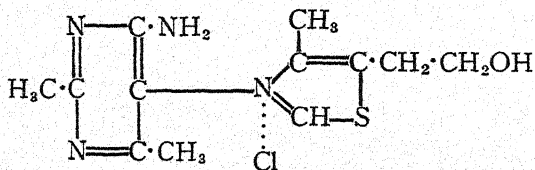


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The structure of II was established and confirmed by synthesis and determined to be that of a 4-methyl-5- β -hydroxyethyl-thiazole (Clarke & Gurin):



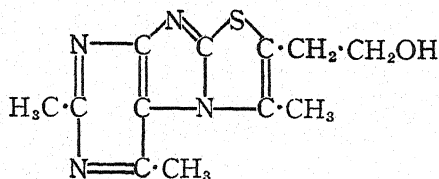
These results point to formula V for vitamin B_1 :

V. Vitamin B_1

In this formula only the positions of the two methyl groups and the thiazolium group in the pyrimidine portion are uncertain. (For measurements of the absorption spectrum see Smakula and Holiday.)

By oxidation of vitamin B_1 a blue-fluorescent substance is formed

[Peters; Kinnersley, O'Brien & Peters (3)]. By oxidizing the vitamin with alkaline potassium ferricyanide Barger, Bergel & Todd (1) obtained a pale yellow, crystalline, blue-fluorescent compound, $C_{12}H_{14}ON_4S$, which was found to be identical with a dyestuff "thiochrome," isolated by Kuhn, Wagner-Jauregg, van Klaveren & Vetter from yeast. By thermal decomposition of vitamin B_1 a blue-fluorescent compound was formed, possessing the formula $C_9H_{10}ON_4$ (Barger, Jansen & Todd). The structure of thiochrome (VI) has been elucidated by Barger, Bergel & Todd (2), Kuhn & Vetter, and Windaus, Tschesche & Grewe (2). This dyestuff is produced by dehydrogenation of the amino group in the pyrimidine portion and the methine group in the thiazole portion of the molecule of vitamin B_1 :



VI. Thiochrome

Vitamin B_1 is not related to the most important coenzymes of carbohydrate oxidation and reduction, i.e., cozymase and codehydrase II (Warburg's coferment).

VITAMIN B_2

Nomenclature.—The nomenclature of this vitamin has been discussed by many authors and various proposals have been made by Chick *et al.*, Elvehjem & Koehn, Guha, György (1, 2), Harris, Jansen, and Euler & Malmberg. In the reviewer's opinion it would be clearest to retain the term vitamin B_2 for lactoflavin (*d*-riboflavin). Thus vitamin B_2 is chemically and physically defined by the structural formula of lactoflavin and it is biologically defined by its growth-promoting action in rats.

Lactoflavin² is not the only preventive factor for dermatitis in rats; probably lactoflavin, together with other factors, has a curative and

² Cf. also this volume, pp. 8, 33, 189. (EDITOR.)

preventive action against dermatitis. Whether there exists besides lactoflavin (B_2) one or several antidermatitis or antipellagra factors is not yet clear. One of these may be called B_6 . Unlike B_2 the latter is not adsorbed by fullers' earth at pH 1 or precipitated by lead acetate at pH 4.0 or 8.0. The vitamin G of the American authors is to be identified, consequently, with B_2 as far as growth-promoting action is concerned. Supplee, Ansbacher & Bender, in researches on vitamin G, found a strict proportionality between yellowish green fluorescence and growth-promoting action.

Vitamin G must be identified with B_6 as far as pellagra-preventive action is concerned.

Distribution of vitamin B_2 .—Since the new differentiation between B_2 and B_6 had not been generally adopted in 1935, research work on distribution shall here be classified, if possible, in accordance with the testing-principle used (B_2 , if growth-promoting action was tested or if chemical identification with lactoflavin was demonstrated). The flavin content of yeast was measured by Pett and by Euler. Heilbron *et al.* isolated lactoflavin from algae; Willstaedt (1) isolated it from orange-agaric (*Lactarius deliciosus*, L.); Kuhn & Kaltschmitt found it in hay and observed also that the flavin of milk is free (not bound to phosphoric acid). Quantitative determinations of flavin in biological materials were also made by Euler, Adler & Schlötzer and Kuhn, Wagner-Jauregg & Kaltschmitt. A flavin-like substance was found by Willstaedt (2) in flax-seed. Euler, Hellström & Adler and also Karrer, Euler & Schöpp examined and isolated the flavin of fish eyes and found it biologically active. Koschura showed that a part of the urine flavin is lactoflavin (concerning urine flavins see also Helmer). Guha & Biswas found a biologically active flavin in animal and vegetable tissues, 70 to 80 per cent of the total flavin being present as flavin enzyme. (For flavin in other animal tissues see Charite & Khanslow.) According to Theorell, Karrer, Schöpp & Frei most of the flavin present in liver is in the form of flavinphosphate. Flavinphosphate promotes the growth of rats as effectively as vitamin B_2 (lactoflavin).

Flavin enzyme ("yellow ferment" of Warburg) is a component of many dehydrogenase systems, and thus takes part in the dehydrogenation of the following substrates (Euler & Adler, not published): hexosemonophosphate, alcohol, glucose, malic acid, lactic acid, citric acid, and hexosediphosphate.

Avitaminosis- B_2 .—If the nomenclature proposed above be ac-

cepted, pellagra can no longer be regarded as avitaminosis-B₂ but rather as avitaminosis-B₆. Nothing is known of avitaminosis-B₂ in humans. In animals, avitaminosis-B₂ causes a loss of weight. Some facts suggest, however, that definite lesions can also attend avitaminosis-B₂. Day has already reported cataract in the eyes of B₂-deficient animals and, furthermore, observed that the growth-promoting and cataract-preventive properties appear to belong to the same factor. Likewise Ray *et al.* found a marked diminution in the indophenol-reducing power³ of the eye lens of rats deficient in constituents of the B complex, but these authors do not consider the abnormality to be due to the deficiency of B₂ or B₁. Bourne & Pyke did not obtain cataract in B₂-deficient rats so regularly as Day.

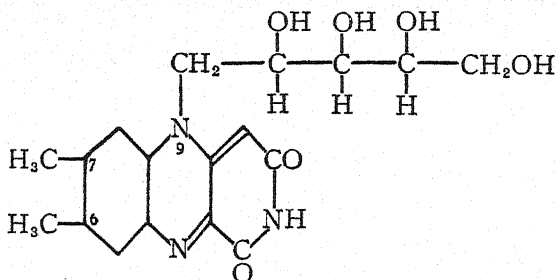
Assay of vitamin B₂.—Concerning the biological test no changes have occurred. The testing method at Stockholm's Biochemical Institute is described by Euler, Karrer, Adler & Malmberg. On the quantitative determination by fluorescence see Vivanco and the critical remarks of Koschara (cf. also Cohen; van Eekelen & Emmerie; Josephy).

Physical and biological properties of vitamin B₂.—The melting point of the best natural and synthetic preparations of vitamin B₂ is 282° (not corrected). (Kuhn, Rudy & Weygand report a melting point of 293°.) The tetra-acetyl compound melts at 238 to 239°. A daily dose of 3 µg.⁴ gives in rats a weight increase of 0.9 gm. per day [Euler, Karrer, Malmberg *et al.*; György (2)]. The specific rotation (Karrer & Fritzsche; Kuhn, Rudy & Weygand) is -96.6° (±4°) for a 0.15 per cent solution in 0.05 N NaOH and -90.0° (±5°) for a 0.1 per cent solution. In the presence of boric acid, lactoflavin is dextrorotatory ([α]_D²⁰ = 350°, Kuhn & Rudy).

Chemistry.—The constitution of the isoalloxazin nucleus in vitamin B₂ (lactoflavin), as elucidated by the classical researches of 1934 by Karrer, Kuhn, Stern and their coworkers, has been confirmed by synthesis in 1935. The structure of the carbohydrate group was first considered by Kuhn and coworkers as an *l*-arabityl residue, but was later proved to be a *d*-ribityl residue (Euler, Karrer, Malmberg *et al.*; Karrer, Schöpp & Benz; Kuhn, Reinemund *et al.*). The following structural formula for lactoflavin (VII) is now definitely accepted:

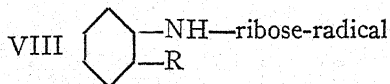
³ Concerning the relation "diminished indophenol-reducing power and cataract" see vitamin C.

⁴ 1 µg. = 1 microgram = 0.001 mg. = 1 γ.

VII. 6,7-dimethyl-9-(*d*-1'-ribityl)-isoalloxazin (lactoflavin)

For the synthesis of *d*-riboflavin, *o*-aminophenylribamine (VIII, $R = NH_2$) is condensed with alloxan by action of acids, substance VIII ($R = NH_2$) being first prepared from the respective nitro compound VIII ($R = NO_2$) or carbethoxy compound VIII ($R = NHCOOC_2H_5$).

Karrer & Meerwein have found a new preparative way to produce



by the action of *p*-nitrophenyl-diazonium-chloride on VIII ($R = H$) and reduction of the azo dyestuff obtained. Great progress in the synthesis of VII was made by the addition of boric acid in the condensation of the ribamine with alloxan as proposed by Kuhn & Weygand; the yields could then attain 95 per cent of the theoretical amount. A history of the synthesis of lactoflavin is given by Karrer, Becker, *et al.*

Isomeres of B₂ and chemically related compounds.—By the same principles of synthesis a great number of related compounds may be synthesized. These differ from lactoflavin by substitution in the isoalloxazin nucleus or in the nature of configuration of the carbohydrate group. It is not possible to relate here all the researches concerning these questions but it is to be noted that besides lactoflavin (VII) other analogous compounds possess vitamin-B₂ activity but in lower degree. The antipode of VII, 6,7-dimethyl-9-(*l*-1'-ribityl)-isoalloxazin, has only a third of the biological activity of VII (Euler, Karrer & Malmberg); the 6,7-dimethyl-9-(*d*-1'-arabityl)-isoalloxazin has, likewise, a biological activity of about a third of that of VI; the antipode, 6,7-dimethyl-9-(*l*-1'-arabityl)-isoalloxazin has no constant activity.

Also lower homologues of lactoflavin have been examined; Karrer, Euler & Malmberg found the 7-methyl-9-(*d*-1'-ribityl-isoalloxazin to be one-half as active as VII, and Karrer & Strong observed the isomeric 6-methyl compound to be active also. Synthetic flavins with xylose as the carbohydrate group were inactive.⁵

Flavin enzyme.—The principal facts concerning the chemistry of the yellow ferment (researches of Warburg & Christian and of Theorell) have been reported in the preceding volume of this *Review*.⁶ The position of the phosphoric acid residue in the natural lactoflavinphosphoric acid has not yet been determined. The natural compound is not identical with a lactoflavinphosphoric acid obtained by Kuhn & Rudy by phosphorylation of lactoflavin.

Theorell (1) begins his paper with a remarkable historical exposé concerning the discovery of the flavin enzyme and of the work of Karrer and Kuhn and their colleagues on the constitution of flavin. Theorell's measurements of the reversible hydrolysis of the flavin enzyme under different conditions are also presented.

In the flavin enzyme we have the best example hitherto known of the relation between an enzyme and its active (prosthetic) group of vitamin or hormone character. For such enzymes the names "vitazyme" and "hormozyme" were, therefore, proposed (Euler, 1933).

In the second paper Theorell (2) shows that flavinphosphate reacts quantitatively under the influence of light like flavin itself. In the absence of oxygen, flavin and flavinphosphate are much more rapidly destroyed than when oxygen is present; oxygen protects flavin against the destructive effect of radiation.

VITAMINS B₄ AND B₆: ANTIDERMATITIS FACTORS

Harris,⁷ summarising the research of the preceding year, expressed the opinion "that vitamin-B₄ deficiency seems to resemble a state of chronic or persistent deficiency of vitamin B₁, since it can always be cured by the administration of a sufficiently large dose of vitamin B₁." Kinnersley, O'Brien & Peters also consider that vitamin B₁ in excess can act as a substitute for vitamin B₄, or, alter-

⁵ Concerning other synthetic flavins with various substituents (pentoses, hexoses, hexitols) in the 9-position, see the researches of Karrer and coworkers (Euler, Karrer, Malmberg, Schöpp, Benz, Becker, and Frei) in *Helv. Chim. Acta*, **18**, 522 (1935), and of Kuhn and coworkers in *Ber.*, **68** (1935).

⁶ *Ann. Rev. Biochem.*, **4**, 493 (1935).

⁷ *Ibid.*, **4**, 346 (1935).

natively, that vitamin B₄ is present either in the diet or in the rat, i.e., by storage. These authors find after a careful examination of the evidence for vitamin B₄, presented by Reader, that two vitamin-B₄ factors, B_{4a} and B_{4b}, have been proposed. In the reviewer's institute in Stockholm Miss Malmberg (unpublished experiments) has not been able to restore growth in rats with B₁ and lactoflavin (cf. Euler, Karrer & Malmberg, 1935). Elvehjem & Arnold find, in accordance with Harris, that if the rats showing vitamin-B₄-deficiency symptoms are given vitamin B₁ the symptoms gradually disappear, "but the animals do not show the dramatic response obtained in animals with uncomplicated polyneuritis."

This corresponds with the results of Chick, Copping & Edgar, that pure hepatoflavin or lactoflavin when added as a small daily dose (12-20 γ) to a basal diet deprived of vitamin B₂, but containing vitamin B₁, was found to restore growth in rats to a small extent (increase of weight up to 6 g. weekly). In order to attain normal growth (10-12 g. weekly) a small daily dose of the supplementary material (equivalent to 0.5-1.0 g. dry yeast) was also necessary.

These authors provided, as the missing supplement for lactoflavin, a yeast extract from which the B vitamins had been adsorbed by treatment with fullers' earth or, when vitamin B₁ was supplied, a concentrate made from bakers' yeast by Peters' process. György's earlier conclusion that the heat-labile "vitamin B₄" was the missing factor has, however, recently (1) been abandoned by him in favor of the idea that the supplement needed by flavin, which is present in Peters' preparation, is a new heat-stable component of the vitamin-B complex, which he calls vitamin B₆; he suggests that it may be identical with the factor "Y," described by Chick, Copping & Roscoe. Chick and her coworkers and György are of the opinion that vitamin B₆ together with flavin forms a combination having the full biological action formerly attributed to vitamin B₂.

In a recent publication on the vitamin-B₂ complex, Birch, György & Harris came to the conclusion that the human pellagra-preventing ("P.P.") factor is different both from vitamin B₆ (hitherto called the "rat pellagra" factor) and from lactoflavin; the P.P. factor should therefore be regarded as a third component of the vitamin-B₂ complex. "Rat pellagra" is by these authors not regarded to be analogous to human pellagra but is a separate entity. The so-called "chicken pellagra" of Elvehjem & Koehn appears to be distinct from vitamin-B₆ deficiency.

Experiments on the distribution of vitamin B₆ are reported by

György (4). He found fish muscle (herring, salmon, and haddock) to be a rich source of B₆ (lactoflavin is practically absent from this material).

On the chemical composition and structural formula of B₆ nothing is known as yet. B₆, as well as B₂, is susceptible to inactivation by visible light [György (5)].

Interesting histological researches on skin lesions, dermatitis, and pellagra in animals have been carried out by Krieger Lassen. Euler & Malmberg summarize their experiments as follows: The forehead skin of rats, fed on a basal diet plus 10 µg. of lactoflavin plus flavin-free heated yeast extract, shows marked histological alterations in comparison with normal skin. These observations indicate that the normal mixed diet contains substances which were not present in the experimental diet used.

Our knowledge of the antidermatitis factors is still very incomplete; it seems, however, that besides lactoflavin two factors are needed for restoring normal condition of the skin: these hypothetical substances may be the vitamins B₆ and H (see also Moncorps).

VITAMIN B₈

Pigeons on a polished rice diet, supplemented with vitamin-B₁ concentrates alone, show, according to Carter & O'Brien, an early rise in weight which may be followed by a slight decline suggesting deprivation of a stored factor or factors. Comparison of the food intakes of birds on such a diet and on whole wheat shows that the amount of protein ingested is inadequate. This deficiency of protein in polished rice can be remedied by the addition of caseinogen or gluten to the basal diet whereby a recovery in weight is produced. The extent of this recovery varies with the degree of depletion of a stored vitamin factor, B₈.

VITAMIN C

The physiological effects of the different components of the vitamin-B complex are hitherto not wholly defined in detail. Vitamin C, on the contrary, is clearly characterized by its antiscorbutic power: hemorrhages and dental defects regularly appear if ascorbic acid is the only vitamin absent from the diet.

Distribution.—A great number of researches demonstrate the wide distribution of vitamin C and show, also, that it may be found in relatively high concentration in the tissues and secretions of plants

and animals. Bourne & Allen found in lower organisms a substance capable of reducing a reagent which, in higher forms of life, is used as a specific test substance for vitamin C. It may, therefore, be that "the very existence of living protoplasm is dependent upon the presence of this vitamin."

In plant tissues Giroud, Ratsimamanga & Leblond have reported a direct proportionality between the content of vitamin C and carotenoids or chlorophyll (see also Randoin, Giroud & Leblond). Growing plant tissue has always a high vitamin-C content (Hausen). The rôle of the vitamin in connection with plant growth has been studied by Hausen and Havas.

Reductones, compounds able to reduce dichlorophenolindophenol under the same conditions as vitamin C (Euler & Martius), are present in yeast. Substances of reductone character, but not identical with vitamin C were isolated from the metabolic products of *Penicillium Charlesii* by Clutterbuck, Raistrick & Reuter. Much vitamin C was found by Ahlberg in dill and parsley. Other results concerning the vitamin-C content of food plants are reported by the following: Ahmad; Aschehoug; Caserio; Cultrera; Damodaran & Srinivasan; Ghosh & Guha; Guerrant, Rasmussen & Dutcher; Izumrudowa; Janovskaja; Jarussowa; Kedzov; Levy & Fox; Lo; Matzko; Minz & Sirianni; Pfützer & Pfaff; Pochino; Rygh *et al.*; Shepilevskaja.

Attention may be drawn to Virtanen's interesting results on the influence of plant nutrition on the vitamin-C content of beans and peas.

Many interesting facts have been reported concerning the distribution of vitamin C in animal tissues and liquids. The high content of the lens and aqueous humour in ascorbic acid, referred to in the preceding volume,⁸ is the subject of renewed investigation. Euler & Malmberg (1) found in the lens of normal rabbits and guinea pigs 0.16 mg. of ascorbic acid per gm. If there is a dietary deficiency of vitamin C the level in the lens diminishes to 0.005 mg. per gm. of lens tissue in guinea pigs and to 0.10 mg. per gm. in rabbits. Large doses of vitamin C increase the content in the lens to 0.26 mg. per gm. In rats the vitamin-C content of the lens is much lower (0.02 mg. per gm.). In the human normal lens an average was found of 0.31 mg. of ascorbic acid per gm. In cataract this value is diminished to 0.05 mg. per gm. (cf. also Nakamura & Nakamura and Weinstein; the last-mentioned author shows that vitamin C is still present in the eye

⁸ L. J. Harris, *Ann. Rev. Biochem.*, 4, 351 (1935).

capsule when it has disappeared from the lens). According to the opinion of Müller the lens is able to transform hexoses into ascorbic acid. Ray, György & Harris also found the dichlorophenolindophenol reducing-power of the lens greatly diminished in rats fed with certain experimental diets lacking constituents of the vitamin-B complex. This disorder could be cured by raw egg white. Other researches on the vitamin-C content in the eye have been carried out by the following: Bietti; Bietti & Carteni; Goldmann & Buschke.

When guinea pigs are on a diet containing ascorbic acid the substance is selectively absorbed by the tissues of the intestinal tract, principally by those of the small intestine (Zilva). With regard to vitamin C and glutathione in the tissues of normal and tumor-bearing animals, see Woodward. Localization of vitamin C in the teeth has been studied by Demole, Cahen & Pfaltz. A series of researches concerns the vitamin-C content of the brain [Malmberg & Euler; Plaut & Bülow (1); Plaut, Bülow & Pruckner; Plaut & Stern]. Ascorbic acid occurs here in high degree. Most was found in the embryo and infant (these observations are in accord with the high capillary resistance in infants). The vitamin-C content of hypophysis was determined by Giroud, Leblond & Ratsimamanga (see earlier work of Euler). Plaut & Bülow (1, 2) have reported investigations on the cerebrospinal fluid (see also Tatsumi *et al.*). Here the ascorbic acid is present only in the reduced form and vitamin-C deficiency of short duration has no influence on the ascorbic acid content. If given over a long time a diet particularly rich or poor in vitamin C has a very distinct influence on the ascorbic acid level in the cerebrospinal fluid. When the metabolic rate is high (malaria, hyperthyroidism) the ascorbic acid content of the cerebrospinal fluid is diminished.

In blood serum the vitamin-C content is low. The greatest amount (1.9 mg. per cent) was found in horse serum by Schroeder (1). Gabbe found in human serum 0.14 to 1.21 mg. per cent. Van Eekelen, Emmerie, Josephy & Wolff reported that vitamin C in blood is equally distributed between the plasma and erythrocytes. This result was confirmed by Schneider & Widmann. Neuweiler (1) determined the ascorbic acid in the blood of the umbilical cord. Human milk contains 4 to 7 mg. per cent of ascorbic acid, that is to say five to six times as much as cows' milk [Neuweiler (2)]. With reference to vitamin C in milk powders see Renner.

The first work on the vitamin-C content of urine was done by Euler & Klussmann (1933). These experiments, in addition to a

review on vitamin C, are referred to in a preceding volume.⁹ In recent researches Euler & Malmberg (2) found that Swedish city-dwellers excreted an average of 25 mg. per day. In the rural population of Sweden this average is reduced to 5 to 11 mg., in agreement with the fact that in many provinces of Sweden the rural population has a diet particularly poor in vitamin C [Euler & Malmberg (3)]. In German city-dwellers Drigalski (1) found an average excretion of 50 mg. per day. The titration of ascorbic acid with iodine, as employed by this author, is quite practicable in weak solutions but does not seem to be suitable for the assay of urine. For further researches on the ascorbic acid content of normal urine see Bezssonoff, Gabbe, Ippen, Rohmer *et al.*, and the monograph of Leblond. As regards vitamin C in the urine of animals see Euler & Malmberg (2).

The vitamin-C content of urine has also been determined in pathologic conditions. In diabetes, no distinct relation between the severity of the disease and the vitamin-C level in the urine could be detected [Euler & Malmberg (4); Drigalski]. Some diabetic urines were, nevertheless, absolutely free from ascorbic acid. For the excretion of vitamin C in other pathologic conditions see Finkle; Harde, Rothstein & Ratish; Pinotti; and Schroeder (2). With reference to the influence of general ether anesthesia on the urinary excretion of vitamin C see Zilva (1). Harris & Ray have developed a very interesting method of diagnosis for vitamin-C subnutrition by urine analysis which has been applied by Abbasy, Harris, Ray & Marrack.

Assay of vitamin C.—Various accounts of the testing methods have been published (Bomskov; Scheunert & Schieblich). A method for the histochemical detection (silver nitrate test) of ascorbic acid was worked out by Giroud & Leblond and has been described in numerous papers. The method was also used by Bourne & Allen. The interfering action of glutathione in the silver nitrate test was discussed by Svrbely. Most of the determinations of vitamin C have been made by the dichlorophenolindophenol titration (Tillmans' method). By titration at pH 2.5 to 3.0 it is possible to avoid the reaction of cysteine and glutathione. In the precipitation of cysteine and glutathione by mercuric acetate, as proposed by Emmerie & van Eekelen,¹⁰ adsorption of vitamin C may occur; erroneously low results may therefore be obtained [Euler & Malmberg (1); Fischer]. By the mercuric acetate precipitation, thiosulfate, which

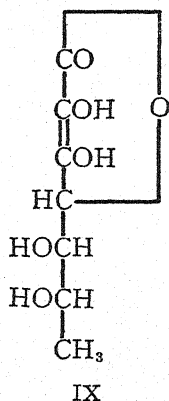
⁹ *Ann. Rev. Biochem.*, 3, 272 (1934).

¹⁰ Cf. *ibid.*, 4, 350 (1935).

may interfere, is also removed [van Eekelen (1)]. Fujita & Iwatake propose the use of metaphosphoric acid for acidification in Tillmans' method. They show that trichloroacetic acid itself reduces the indicator. For other observations concerning the titration with dichlorophenolindophenol see the following: Ahmad; Deviatnin & Doroschenko; McHenry & Graham; Ray, György & Harris. Concerning the influence of oxidases in the determination see van Eekelen (2); for the determination of minute quantities see Glick. Contrary to the data of Jonnissian, the Tillmans indicator is not reduced by fructose and arabinose (Bacharach & Glynn). Tillmans' method is criticized by Bezssonoff (1) but his arguments cannot be considered convincing in view of the numerous well-founded results obtained with this method. Euler & Burström have compared the results obtained by the Tillmans and Bezssonoff (2) methods in urine and could not endorse the criticism of Bezssonoff. On the contrary they found that ascorbic acid could be titrated with Bezssonoff's reagent in pure aqueous solution but not in urine because other reducing substances, present therein, interfered. For a method of titration of ascorbic acid in the presence of much glutathione see Pfankuch. A determination of vitamin C with phospho-18-tungstic acid has been worked out by Medes. In the researches of Plaut, Bülow & Pruckner ascorbic acid was also determined spectrophotometrically. Color reactions for ascorbic acid, based on the reduction of gold chloride (AuCl_3) or of selenium oxide (SeO_2) have been proposed by Emmerie.

*Chemistry.*¹¹—The hydroxyl group in vitamin C is most responsible for the acid properties of the vitamin (Birch & Harris; Micheel & Schulte; Haworth *et al.*; Reichstein *et al.*). A synthesis, starting from *d*-glucose, has been described by Maruyama. The essential condition for the antiscorbutic activity in the ascorbic acid group is the *d*-configuration of the fourth carbon atom, as pointed out by Reichstein *et al.* (cf. also Dalmer & Moll). Reichstein, Schwarz & Grüssner found, in accordance with this, that 6-methyl-*l*-arabo-3-keto-hexonic acid lactone (IX) is antiscorbutically active (one-fifth of the activity of ascorbic acid itself). The behavior of *l*-ascorbic acid and chemically related compounds in the organism was studied by Zilva (2, 3). Daily doses of 20 mg. of *d*-ascorbic acid had no antiscorbutic activity in guinea pigs; *d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid were also inactive.

¹¹ Cf. also this volume, p. 86. (EDITOR.)



An extremely powerful enzyme which specifically oxidises ascorbic acid to the dehydro compound was found in the pericarp of *Cucurbita maxima* by Tauber & Kleiner. This oxidase is stable to aeration, carbon monoxide, and small amounts of potassium cyanide, but is irreversibly inactivated by hydrogen sulfide or by tryptic digestion. Ascorbic acid is very sensitive to oxidation by oxygen and small amounts of heavy metal catalysts (Euler, Myrbäck & Larsson); when metallic catalysts are absent from the solution ascorbic acid is very resistant to oxygen. It should be mentioned that erythrocytes (intact and laked) exercise a marked protective action on ascorbic acid (Kellie & Zilva); this is in agreement with observations by other authors (Mawson) on the protective action of animal tissues on ascorbic acid.

Biosynthesis.—Hypotheses concerning the synthesis of ascorbic acids by animals which do not require vitamin C in the diet were discussed in the preceding volume of this *Review*.¹² Guha & Ghosh completed their earlier work by experiments *in vivo* and found, five hours after the subcutaneous or intravenous injection of mannose (20 mg.), an augmentation of the ascorbic acid content of rats. They found, also, that embryonic guinea-pig tissue, ovarian tissue (early stage of development) of the pregnant guinea pig, and ovarian tissue of the adult non-pregnant monkey are capable of converting mannose into ascorbic acid *in vitro* on incubation for five hours at pH 7.4. These *in vitro* results could not be confirmed by Euler, Gartz & Malmberg; no experiments comparable to the studies *in vivo* were conducted. A conversion of mannose into ascorbic acid in the human

¹² *Ann. Rev. Biochem.*, 4, 352 (1935).

infant is also reported by Banerjee: there was a greatly increased excretion of ascorbic acid after a daily dose of 1 gm. of mannose. Mosonyi suggests that vitamin C can also be synthesized by the organism from methylglyoxal and acetoacetic acid with 3-hydroxy-acetylacetone (Henzes ketol) as an intermediary product.

Vitamin C and enzyme reactions.—Ascorbic acid activates the catheptic enzymes of the liver. Reductone (Euler & Martius) and reductic acid (Reichstein & Oppenauer) also exert an activating influence on these enzymes, but to a lesser degree; dehydroascorbic acid is inactive (Karrer & Zehender; Euler, Karrer & Zehender). Maschmann & Helmert and Purr showed that papain is activated by ascorbic acid. As shown by Schaaf vitamin C does not prevent the tyrosinase reaction (tyrosine \rightarrow dihydroxyphenylalanine); however, the reaction dihydroxyphenylalanine \rightarrow melanine, is checked by vitamin C (Abderhalden). In accordance with these facts the pathologic pigmentation in Addison's disease can be lessened by ascorbic acid therapy (Szent-Györgyi; Morawitz). Concerning the biochemical relations between vitamin C and the other constituents of the adrenal glands see Bersin *et al.* The coagulation of blood is accelerated by vitamin C (Kühnau). Pre-treatment of β -malt-amylase with reduced ascorbic acid results in inactivation of the enzyme (Hanes); a measurable time is required for the maximum effect.

Therapeutic use of vitamin C.—The use of vitamin C in human therapy has greatly increased for two reasons: (a) hypovitaminosis-C (a state in which definite scorbutic symptoms are not manifest, but in which the vitamin-C content of the organism is low) has been shown to be very common in human beings; (b) ascorbic acid has also become a specific therapeutic agent in many cases of blood- or vascular disease. The results of vitamin-C therapy are described in a series of clinical publications by Stepp and his coworkers, but also by other authors. A survey of such therapeutic applications is given in a publication by Willstaedt.¹⁸

Vitamin C and infection.—Since the fundamental work of E. Mellanby on the anti-infectious properties of vitamin A appeared it must be regarded as experimentally demonstrated that various infectious diseases are attributable to lack of vitamin A. However, the problem of the relation between susceptibility to infections and the intake of vitamins is, from different points of view, a very complicated

¹⁸ I am also indebted to Dr. Willstaedt for valuable assistance in collecting literature for this review.

one, especially because most of the known vitamins seem, to some extent, to have the power of preventing local infections.

Many observations are published indicating anti-infectious effects of ascorbic acid. But as we have at present no information concerning the biochemical basis for the different anti-infectious effects it would be of little value to review the many cases in which healing of infectious diseases, after treatment with ascorbic acid, has been alleged. Only the results of King & Menten as well as Polónyi may be mentioned, which show that the resistance of guinea pigs against diphtheria toxin is increased by large doses of ascorbic acid given *per os* or by injection.

Effect of vitamin C on anemia.—Even here the data which have appeared in the literature are contradictory and are not sufficiently defined to permit rigorous conclusions. It is known that erythrocytes contain vitamin C and that avitaminosis-C induces grave cytological alterations in the bone marrow and a retarded formation of erythrocytes (Mettier & Chew). Similar results are reported by Böger & Martin and by Seyderhelm & Grebe.

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THE FAT-SOLUBLE VITAMINS*

By E. V. McCOLLUM

The Johns Hopkins University, Baltimore, Maryland

VITAMIN A

Carotene as the international standard of vitamin A.—Hume & Chick (1) have edited certain significant technical information, not available elsewhere, concerning the properties and use of the international vitamin-A standard. The bulletin is intended for the information of competent workers already practiced in the usual methods for the quantitative estimation of the vitamin. Certain data from this important compilation are here reported.

Baumann & Steenbock (2) studied the effect of light, temperature, and chemical stabilizers on the stability of carotene in several solvents. Refined cottonseed oil (Wesson) gave the best results; followed by sesame, coconut, olive, corn, and crude wheat-germ oil in the order given. Hydroquinone in a concentration one-tenth that of carotene did not increase its stability. Ethyl laurate and ethyl sebacate permitted rapid deterioration, but addition of hydroquinone improved the stability of dissolved carotene. In cottonseed oil there was a loss of 26 per cent within a month when stored at room temperature; storage should be in the dark and at 0° C. Rancidity in the oil did not appear to be detrimental to the stability of dissolved carotene. Biological tests were not reported. McDonald (3) also found Wesson cottonseed oil the most satisfactory solvent. Turner (4) found that 0.1 per cent hydroquinone added to 0.2 per cent carotene delayed the fading of color of carotene dissolved in ethyl laurate and olive oil. He found that half-faded solutions gave values in rough biological tests of about half those of the newly-made solution.

Culhane Lathbury & Greenwood (5) obtained in biological tests very different results with comparable doses of carotene administered in arachis and coconut oils, and also when different samples of these oils were used. Their tests were not controlled as to rate of color fading, but the solutions were kept at low temperatures in full bottles, so their conclusion that different samples of the same oil, as solvents, may cause variation in the degree to which carotene may be utilized by an animal, appears to be trustworthy.

* Received January 6, 1936.

A group of English workers collaborated in physical and biological tests made on the specimen of carotene used as the international standard from 1931 to 1935, and on a sample of β -carotene, prepared by Karrer, which the International Conference of 1934 decided should supersede it. Those who participated in the physical tests were: K. Culhane Lathbury & G. N. Greenwood, I. Smedley-MacLean & E. M. Hume, and T. Moore. The biological tests were contributed by F. J. Dyer, K. M. Key & K. H. Coward; K. Culhane Lathbury & G. N. Greenwood; and E. M. Hume & H. Henderson Smith. From these studies it is concluded that maintenance or loss of color by a given solution of carotene appears to be a trustworthy index of its maintenance or loss of biological value.

Of the substances examined as possible solvents for the 1931 international standard carotene, coconut oil was the most favorable, in that carotene was well utilized from it, as judged by biological tests, and comparatively stable in it, as judged by colorimetric tests. Three samples of coconut oil from different sources were examined biologically and colorimetrically. With one exception only small differences were detected in the biological value of the dissolved carotene when adequate precautions were taken to preserve its stability during biological experiment. Definite differences in the stability of carotene in the three oils, however, became apparent when the solutions were exposed to conditions which promote instability. There was not sufficient evidence to determine the cause of the small differences in biological value, though potential differences in stability may be suspected. Addition of hydroquinone in adequate concentration exercised a marked stabilizing influence, as judged by color, on carotene in solution in several samples of coconut oil, even in a less favorable specimen. It was on the basis of these studies, which were communicated to the 1934 International Conference on Vitamin Standardization, that a decision was reached to issue the standard in future as a solution of carotene in coconut oil, with addition of hydroquinone.

The above group of collaborators also studied the relation between the vitamin-A activity of the 1931 standard (a product obtained by recrystallization of a mixture of samples of carotene prepared in eight different laboratories in seven different countries) with pure β -carotene. The 1931 standard must have consisted of β -carotene with no significant admixture of α -carotene, since there was no dextro-rotation, but contained a certain amount of inactive material. Karrer prepared for the National Institute for Medical Research

about a gram of pure crystalline β -carotene, and the 1931 standard was compared by biological tests with this material by Coward, by Culhane Lathbury, and by Hume & Smith. The dose of β -carotene equivalent to 1 $\mu\text{g.}^1$ of the international standard carotene was found by these observers to be 0.57 $\mu\text{g.}$, 0.66 $\mu\text{g.}$, and 0.50 $\mu\text{g.}$, respectively, the average value being 0.58 $\mu\text{g.}$ Using another sample of β -carotene, E. M. Nelson found 0.66 $\mu\text{g.}$ equivalent to 1 $\mu\text{g.}$ of the international standard. The average value of 0.6 $\mu\text{g.}$ was accepted by the 1934 International Conference as the figure which should be adopted.

Moore (1), using a Lovibond tintometer, compared the intensity of yellow color of the 1931 standard carotene and the pure β -carotene used in the biological tests by the English workers (Karrer). He found 1,300 yellow units per mg. for the standard and 2,000 for the β -carotene, indicating that the standard contained about 65 per cent of carotene. Dann (1) made a spectroscopic estimation, comparing the extinction coefficient of the peak of the main absorption band at 450 m μ in the spectra of the 1931 standard and the β -carotene (Karrer). For the wave length 450 m μ , $E_{1\text{ cm.}}^{1\text{ per cent}}$ was 1,520 for the international standard and 2,495 for β -carotene, indicating that the 1931 standard carotene contained 60 per cent of carotene. Early in 1935 Morton & Gillam (1) tested the 1931 standard carotene for direct absorption and observed $E_{1\text{ cm.}}^{1\text{ per cent}}$, 463 m μ , to be 1,500 in chloroform, as against 2,200 for pure β -carotene. The antimony-trichloride color test showed $E_{1\text{ cm.}}^{1\text{ per cent}}$, 590 m μ , to be 331 and 420 for the standard carotene and the β -carotene, respectively. Chromatographic analysis showed that about 15 per cent of the total pigment of the 1931 standard carotene was different from β -carotene. The results indicate that the β -carotene content of the 1931 standard was about 65 per cent. These investigations extend and confirm earlier work in establishing the equivalence of 1 $\mu\text{g.}$ of the 1931 standard carotene, and of 0.6 $\mu\text{g.}$ of pure β -carotene.

Spectrophotometric method of estimating vitamin A by means of the extinction coefficient of 328 m μ .—Morton & Heilbron [1928 (6)], Drummond & Morton [1929 (7)], and Coward, Dyer *et al.* (8) laid the foundation for the estimation of vitamin A by measurement of the intensity of absorption at 328 m μ . In oils or concentrates containing both vitamin A and carotene this procedure might be used

¹ 1 $\mu\text{g.} = 10^{-6}\text{ gm.} = 0.001\text{ mg.} = 1\text{ }\gamma$.

to measure that portion of vitamin A present; the portion of vitamin value referable to carotene might then be measured by separate spectroscopic tests of the absorption in the visible region, as suggested by Morton & Heilbron [1930 (6)]. At the request of the vitamin-A subcommittee, R. K. Callow, S. K. Crews, F. J. Dyer, N. Evers & W. Smith, R. J. Macwalter, T. Moore & W. J. Dann, and R. A. Morton & J. R. Edisbury collaborated in examining different samples of cod-liver oils for (a) the degree of concordance obtained by different workers, (b) the materials to which the test could be applied, (c) the solvent in which the material should be examined, (d) the need or otherwise of first saponifying the material and the best method of doing so, and (e) the relation between the values for vitamin-A potency obtained by spectrophotometry and by biological tests. The workers determined $E_{1\text{ cm.}}^{1\text{ per cent}}$, 328 m μ , for three samples of cod-liver oils, using their own methods and instruments. When the reports were compared and certain preliminary conclusions were arrived at, three other samples of cod-liver oils were circulated, the workers being asked to unify their technic in certain respects. For the details of the results obtained the compilation of Hume & Chick (1) must be consulted. The results obtained in seven different laboratories for six cod-liver oils showed: estimations on cod-liver oils should be made on the unsaponifiable fraction of the oil. Cyclohexane or ethyl alcohol and not chloroform should be used as the solvent. Only a method of saponification approved for the purpose should be used; three such methods are described. When these precautions were observed the results of different laboratories were reasonably concordant.

The results of estimating vitamin A in the above materials by biological assay in different laboratories are also reported and used as a basis for arriving at a factor for converting $E_{1\text{ cm.}}^{1\text{ per cent}}$, 328 m μ , into international units of vitamin A per gram. The figure for this factor recommended by the vitamin-A subcommittee, and accepted by the International Conference (1934), was 1,600. Reasons are set forth for regarding this factor as provisional, and Hume & Chick express the view that it may be retained as at present for practical purposes.

Hume & Chick (1) also gave the values obtained, in nine different laboratories, in biological tests of the United States Pharmacopoeia Reference cod-liver oil (Oil X) for vitamin-A potency. These values varied from 2,350 to 3,725, the average being 2,998 International Units per gram. The potency assigned to this oil, which has been

adopted as the Subsidiary International Standard for Vitamin A, is 3,000 per gram.

Absorption and utilization of carotene and vitamin A.—Drummond, Bell & Palmer (9) studied the absorption of vitamin A and of carotene in a patient suffering from effusion of chyle into the thoracic cavity caused by pressure on the thoracic duct from a new growth. The carotene and vitamin-A content of the fluid were studied before and after oral administration. A relatively small proportion of the carotene administered could be accounted for by the pigment found in the chylous fluid, whereas in the case of vitamin A absorption was nearly complete. Little of the carotene, and none of the vitamin A could be extracted directly from the chylous fluid with ether, but treatment with alcohol rendered the carotene easily extractable. The vitamin administered as the free alcohol was found in the lymph mainly in the esterified form, and it is thought probable that the linkage with fatty acids during passage through the intestinal walls accounts for the much higher coefficient of absorption, as compared with that when carotene was given. Observations on the chylous fluid showed that over a range of reaction much wider than that encountered in body fluids, no trace of either carotene or vitamin A passed through a dialyzing membrane. Both substances appeared to be in colloidal form and closely associated with the highly dispersed fat.

Greaves & Schmidt (10) observed that female rats deprived of vitamin A, and with internal bile fistulas (having cannulas connecting the bile duct near the liver with the upper part of the descending colon) were compared with untreated, deficient rats in regard to their ability to absorb carotene and vitamin A. The state of the vaginal smear was the criterion of absorption. Untreated animals responded to carotene therapy in six days or less. Treated rats, which usually died a few days after operation, did not respond to oral administration of carotene, but responded to subcutaneous injections. Oral administration of carotene became effective in treated animals when glycodeoxycholic or deoxycholic acid was simultaneously given, suggesting that these bile acids function as carriers of carotene across the intestinal wall. Variation in the fat content of the diet of 3 to 23 per cent did not materially influence the absorption of carotene fed orally. They confirm previous work in finding that vitamin A, as opposed to carotene, when fed orally, was absorbed by a rat with an internal bile fistula in amount sufficient to correct the vaginal smear picture.

Greaves & Schmidt (11) found that female rats deprived of vitamin A and rendered icteric by double section and ligation of the common bile duct at a level near the liver, could not absorb carotene orally, as shown by failure of response in the character of the vaginal smear. Rats suffering from liver damage caused by phosphorus administration appeared to possess a decreased ability to transform carotene to vitamin A, but no such effect was observed when rats were treated with benzene, chloroform, or carbon tetrachloride which produced mild liver damage. Icteric rats appeared to be capable of absorbing preformed vitamin A.

Wendt (12) states that the normal human serum content of carotene and vitamin A varies widely, averaging 8.6 and 1.4 units respectively. On peroral administration these values rise rapidly to reach a maximum not further increased by prolonged administration of large quantities. Both substances are stored in large quantities. In rabbits, vitamin A causes hyperlipemia and hyperlipoidemia; in dogs, no effect on blood cholesterol, cholesterol esters, or phosphatide content could be demonstrated.

Davies & Moore (13) state that when adult rats, already possessing high liver reserves of vitamin A, were given a diet containing massive doses of vitamin-A concentrate, the liver reserves reached levels representing a supply sufficient to satisfy the theoretical requirements of the rat for about a century, if used at a rate corresponding to the minimum physiological requirements. When subsequently restricted to a diet free from vitamin A, a rapid elimination of the vitamin from the liver took place. After twelve weeks a condition of stable storage of vitamin A appeared to have been attained, since no further fall occurred during a succeeding twelve weeks on an A-free diet. Superfluous reserves of the vitamin, held in the liver, may be eliminated at a much greater rate than that required to satisfy the known physiological requirement. In earlier studies in which carotene was used instead of a concentrate, such a rapid elimination of vitamin A from the liver was not observed. The cause of this requires further study.

Vitamin A and the common cold.—Cameron (14) concludes that:

Vitamin A, in the form of cod-liver oil, haliver oil, and carotene solution reduced the average number of colds little, if at all, in a group of over 200 students studied from October to April inclusive.

The average duration of colds, however, was reduced by such treatment by from five to ten days per student, which, on statistical analysis, shows a significant difference.

Approximately 60 per cent of the students receiving such treatment showed some improvement in number or duration of colds.

Approximately 50 per cent reported improvement in endurance, sense of well-being, appetite, or skin eruptions.

The average vitamin-A content of the diet of these volunteers was 4,327 units (old U.S.P.).

Students receiving 5,000 or more units of vitamin A in their diet, alone, or in the diet plus the oil taken, showed a decrease of about 0.4 in the average number of colds and of about five days in the average duration, over the figures for the students receiving less than 5,000 units daily. Since the diet of the majority of the students averaged 3,000 or more units of vitamin A, which has been given as adequate, it is evident that these students were not suffering from an actual deficiency of vitamin, but that the larger amounts, nonetheless, produced improvement in colds.

A group of nine girls receiving 5,000 additional units of vitamin A in the form of food, also gave a low figure for incidence and duration of colds, showing that an increased intake of vitamin A from any source reduces the average duration of colds.

The average number of colds per person in the control groups, 2.5, 2.9, 2.8, is comparable to those obtained in other studies, while the seasonal distribution is the same.

It is concluded that vitamin A is a factor in reducing somewhat the severity and duration of colds in young adults, although not a specific against colds, and not to be considered a cure for colds.

Converse, Wiseman & Meigs (15) studied, over a three-year period, the relation of the color and vitamin-A content of butter and skim milk to the feed of the cow. The following tables show the carotene content of butter as influenced by feed.

TABLE I
CAROTENE IN BUTTER AS AFFECTED BY VARIOUS KINDS OF ROUGHAGE

Kind of Roughage in Ration	Carotene Content			
	Holstein	Jersey	Average	
	<i>µg. per gm.</i>	<i>µg. per gm.</i>	Holstein	Jersey
Pasture (spring and fall)*	3.6 to 7.0	8.1 to 17.0	5.8	11.4
Pasture (midsummer 1934)†.....	1.2 to 3.2	3.6 to 4.2	2.2	3.9
No. 3 timothy hay with 20 pounds of carrots	2.4 to 2.5	3.9 to 5.9	2.5	5.1
No. 1 alfalfa hay.....	1.1 to 3.1	2.7 to 3.7	1.9	3.2
No. 1 timothy hay.....	0.8 to 1.6	0.8 to 2.5	1.2	1.3
No. 3 alfalfa hay.....	0.5	0.5 to 1.1	0.5	0.8
No. 3 timothy hay.....	0.2 to 0.6	0.4 to 0.6	0.4	0.6

* Cows got some hay in addition to pasture.

† Rainfall during July and August 1934, about 65 per cent normal.

TABLE II

RELATION OF CAROTENE IN THE FEED TO THE CAROTENE IN THE BUTTER
(SAME HOLSTEIN COW FED THIRTY DAYS ON EACH RATION)

Roughage of the Ration	Estimated Carotene Eaten Daily	Carotene Content of Butterfat
	grams	μg. per gm.
Pasture with alfalfa hay.....	2.36	4.5
Extra green alfalfa hay (15 pounds).....	0.59	3.3
No. 3 timothy hay with 15 pounds of carrots.....	0.64	2.6
No. 1 alfalfa hay (15 pounds).....	0.22	2.0
No. 1 timothy hay (15 pounds).....	0.14	1.6
No. 3 timothy hay (15 pounds).....	0.03	0.4

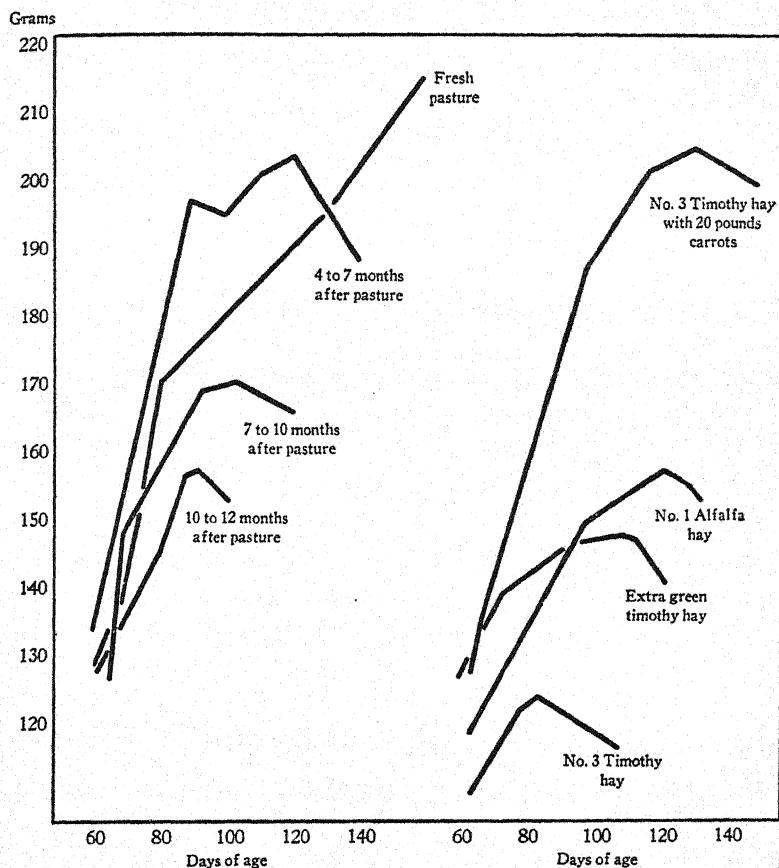
They also studied the vitamin-A content of skim milks by feeding to rats a vitamin-A-free diet supplemented with 40 cc. of skim milk per day per rat. The results are shown in the accompanying curves.

VITAMIN D

Interest has steadily increased in the question of the multiplicity of substances which possess antirachitic properties. The problem is complicated by the fact that absorption and utilization of a substance may be greatly influenced by the medium with which it is administered. Thus, Culhane Lathbury & Greenwood (16), using peanut and coconut oils, obtained very different biological values for carotene when dissolved in these solvents, and also when different samples of these oils were used. A number of facts have now emerged as a result of recent investigations which seem to afford positive proof of the existence of several distinct antirachitic substances.

Bills & McDonald (17) found that when heated with fullers' earth, cholesterol is converted into dicholesteryl ether, which on further heating is degraded into a resin with distinct antirachitic action, being about equal to cod-liver oil, weight for weight. Butyl nitrite destroys the antirachitic property of irradiated cholesterol, but does not reduce the activity of the substance formed on treating cholesterol with fullers' earth. Hence more than one molecular configuration must be capable of exhibiting antirachitic activity.

Recently Yoder (18) has shown that when cholesterol is dissolved in carbon disulfide and treated with sulfur trioxide it is completely dehydrated with the formation of cholesterilene, which is then sulfonated. Cholesterilene itself is inactive but the sulfonic acid derivative is easily of measurable activity. The action of fullers' earth appears to depend upon its sulfuric acid content.



Rat growth curves showing decline of vitamin A in milk after the pasture season. Cows got No. 1 alfalfa hay.

Rat growth curves showing difference in vitamin A of milk produced with different roughages.

(Converse, Wiseman, & Meigs)

Bills (19) showed in 1925 that cholesterol freed from ergosterol by treatment with charcoal could be activated by irradiation. This fact has since been repeatedly confirmed [Jendrassik & Keményffi (20); Kon, Daniels & Steenbock (21); Koch, Koch & Ragins (22); Koch, Koch & Lemon (23)]. Koch and his co-workers made the noteworthy observation that cholesterol which had been freed from ergosterol, and which acquired but low antirachitic potency on irradiation, could be made about twenty-five times more active by heating

it before irradiation. It seems to be established that cholesterol itself is not capable of activation, but that the activity observed in highly purified specimens is due to some transformation product brought about by the drastic treatment to which it is subjected. Schoenheimer (24) suggested that when cholesterol is heated it undergoes simultaneous hydrogenation-dehydrogenation, resulting in a completely hydrogenated and a second highly unsaturated, ergosterol-like substance which is activatable. Since it appears to be demonstrated that ergosterol contains 28 carbon atoms, and cholesterol but 27, it follows that the provitamin in heated cholesterol is not ergosterol.

The discovery by Bills and by Massingale & Nussmeier (25) that irradiated ergosterol, rat unit for rat unit, is about one hundred times less potent for the protection of chicks than for rats (as compared with the vitamin of cod-liver oil), is now well established, and demonstrates that calciferol is different from the vitamin of cod-liver oil. These two sources of vitamin D are different physiologically, and Bills is convinced that alternative explanations of the anomaly are untenable, since they cannot be explained on the basis of presence or absence of vitamin A or on the nature of the oily vehicle.

Bills (26) points out that irradiated ergosterol tends to elevate the blood phosphorus in an animal relatively more when the same degree of protection against rickets is afforded than does cod-liver oil. The response curves of the chicken have a different shape for each of these two vitamin sources. By response curves he means the curves which one obtains when one constructs a graph correlating the percentage of bone ash with the vitamin unitage administered.

Bills has also produced a new antirachitic agent by treatment of ergosterol with ethyl nitrite, and then treating the reaction product with isopropyl amine. There resulted a product which, for the rat, had antirachitic power several times greater than cod-liver oil. Thus the new antirachitic agent was produced by the action of the same reagent which destroys the vitamin of irradiated cholesterol and of cod-liver oil. As an ergosterol derivative it presumably contains one more carbon atom than the vitamin derived from cholesterol, and as a substance which apparently contains nitrogen it differs from vitamin D derived from either ergosterol or cholesterol.

The multiplicity of antirachitic substances is further illustrated by the production by Windaus & Langer (27) of 22-dihydroergosterol by hydrogenating ergosteryl acetate-maleic anhydride, and subsequent thermal decomposition of the reduction product, giving

the acetate of 22-dihydroergosterol, from which the free sterol was regenerated. This sterol, without a double bond in the side chain, exhibited the same absorption spectrum as ergosterol, and became active on irradiation, although less so than ergosterol.

With the discovery that ergosterol on irradiation becomes so highly potent an antirachitic agent, the simplest possible explanation of the distribution and nature of vitamin D was generally adopted, viz., that there is but one substance which possesses antirachitic properties. The view prevailed that the active principle in fish-liver oils is activated ergosterol, and that foods and animal tissues which are capable of activation contain ergosterol. This view is no longer tenable. The first-rate research of Waddell (28) demonstrated, by a comparison of the assays of the activatable provitamin of spinal-cord cholesterol with rats and chicks, that it could not be ergosterol. The nature of this provitamin is still unknown.

The rule of Gerard that the principal sterol of fungi is ergosterol, of the higher plants phytosterol, and of animals cholesterol has been generally accepted as true. The demonstration that many products of animal and plant origin are capable of activation by irradiation with ultraviolet light appeared to necessitate the conclusion that an exception to this rule occurred in the presence in all natural sterols of a small amount of ergosterol. In the light of recent findings this view becomes untenable.

Bills points out that the history of cholesterol unsettles the view that the sterols of the higher plants, sitosterol, stigmasterol, and dihydrositosterol, acquire no activity on irradiation when sufficiently pure, and that the first two should be reinvestigated. Some of the numerous phytosterols have not been studied. It is now evident that the fact that the spectral absorption bands seen in the vegetable sterols look like those of ergosterol is no proof that ergosterol is present, for the same series of bands is shown by 22-dihydroergosterol and by the provitamin D in cholesterol studied by Waddell.

It is well known that ultraviolet rays are effective in protecting chicks against rickets or in inducing a cure when the disease exists. This fact can be hardly brought into harmony with the view that the provitamin in the skin of the chick is ergosterol, since activated ergosterol is of very low potency in this species. Waddell's work shows that the provitamin D of ordinary cholesterol is not ergosterol, and that the vitamin D produced by irradiating ordinary cholesterol is not calciferol.

Hathaway (29) has found that with chickens the vitamin D produced by irradiating heated purified cholesterol is more effective, rat unit for rat unit, than the vitamin D formed by irradiating non-heated purified cholesterol. The former behaves like cod-liver oil, the latter like calciferol. Thus, her vitamin, prepared from Koch's provitamin D, is a distinct form of antirachitic substance.

Bethke, Krauss, Record & Wilder (30) have made a comparison with chicks of the antirachitic efficiency of irradiated milk, milk produced by feeding irradiated yeast to cows, and cod-liver oil, on a rat-unit basis. They found that it required more than ten times as many rat units of vitamin D in the form of yeast milk than in the form of irradiated milk to produce the same degree of calcification of the bones. Vitamin D from irradiated milk and from cod-liver oil were equally efficient. Similar results have been reported by Haman (31).

Bills has assayed the liver oils of twenty-five species of fish using the rat and chick, and finds that although many of these assay essentially like cod-liver oil for these two species, the liver oil of the blue-fin tuna is only about one-sixth as potent, rat unit for rat unit, as is cod-liver oil. Saponification of the oils was carried out, and comparative assays of the non-saponifiable fractions showed the vitamin of tuna-liver oil to be one-seventh as potent, rat unit for rat unit, as the cod-liver oil vitamin for the chick. Notwithstanding Bills' observation that saponification enhanced the vitamin potency of both oils (25 per cent in the tuna-liver oil and 42 per cent in the cod-liver oil), his experimental method and deductions appear to be reliable beyond question, that the natural vitamin D of blue-fin tuna-liver oil is different from that in cod-liver oil. He states that certain liver oils in this series contained an antirachitic substance which was more effective, rat unit for rat unit, for the chick than was that of cod-liver oil.

The accumulated evidence seems to leave no room for doubt that at least seven forms of vitamin D exist, and that the possible number of molecular configurations which may exert antirachitic effects may be very great.

An important contribution to vitamin-D therapy was made by Eliot (32), who eliminated the poor technical quality characteristic of most studies of the comparative values of vitamin D in cod-liver oil and in viosterol by using carefully standardized materials and fairly generous numbers of selected children. She was unable to detect any

appreciable difference, rat unit for rat unit, between irradiated ergosterol and cod-liver oil as an antirachitic agent for children.

Clinical studies with vitamin D.—Rappoport and coworkers (33) administered 4 to 10 drops daily of viosterol, 10,000 X, which contained 920,000 I.U. of vitamin D per cc. (1 drop contained 30,000 I.U.), to 212 subjects suffering from hay fever and asthma, all believed to be allergic cases. Of twenty-two patients who had uncomplicated hay fever, all were relieved to some degree, and fourteen (70 per cent) were rated as "satisfactory" to "complete." Of the asthma patients, forty-one of forty-six (89.2 per cent) had some degree of relief. Fourteen of the number (30.4 per cent) were in the groups "satisfactory" to "complete." In general, it appeared that a pre-seasonal period of ten days' treatment is as effective as a longer period. Combined treatment with viosterol and pollen injections is more effective than either one alone.

Dreyer & Reed (34) have reported a clinical study of sixty-seven cases of arthritis treated with massive doses of vitamin D (viosterol). Their results are shown in Table III.

TABLE III
DISTRIBUTION OF CASES BY TYPES AND RESULTS OF TREATMENT

	Total Subjects	Clinical Improvement	No Improvement	Results Still Uncertain
Atrophic				
Infectious	10	5	4	1
Rheumatoid	24	20	2	2
Hypertrophic				
Degenerative	14	9	2	3
Menopausal	4	1	1	2
Mixed*	3	2	1	0
Miscellaneous	5	1	3	1
Arthralgias	7	6	0	1
	—	—	—	—
	67	44	13	10

* One case, infectious and gonorrheal; two cases, menopausal and rheumatoid.

The initial daily dose was 200,000 U.S.P. units during the first month. In cases where there were no unfavorable symptoms this was increased by 50,000 to 60,000 units per week. In some stubborn cases doses of 600,000 to 1,000,000 units were given.

Gelfan (35) reports that isolated muscles from frogs receiving daily injections of viosterol (92,000 I.U. per frog) showed increased oxygen consumption over controls amounting to 18 to 29.8 per cent.

Reed (36) found that the administration of very large unitage (92,000 I.U. daily) to a rat weighing 166 gm. increased the metabolic rate from 801 ± 39 Cals. per sq.m. per day to an average of 1127 Cals. per sq.m. per day during the following six days. Larger doses (460,000 I.U. daily) caused a lowering of metabolic rate. With toxic doses there was a transient elevation of rate followed by a decline below the original level.

Vitamin-D content of egg yolks.—Branion, Drake & Tisdall (37) determined the average number of Steenbock vitamin-D units per average market-egg yolk to be 8.6 (26.7 I.U.). Five such egg yolks furnish vitamin D equivalent to one teaspoonful of Steenbock's standard cod-liver oil. By the addition of cod-liver oil to the hen's diet the vitamin-D content of the yolks was increased more than threefold. Irradiated ergosterol was relatively inefficiently transferred to the yolk, although massive doses increased the potency of the yolks some 600 times, and medium doses about 35 times. Small doses of activated ergosterol were transferred to the yolks about as efficiently as the vitamin from cod-liver oil. Irradiating the birds with ultraviolet light for twenty minutes daily or exposing them to sunshine during the summer months caused only slight increase in the antirachitic potency of the eggs. Holding eggs in cold storage for eight to eleven months had little or no effect on their vitamin-D content.

Titus & Nestler (38) studied the effects of including cod-liver oil and viosterol in the rations of laying hens. As the concentration of vitamin D in the viosterol was increased there was a pronounced tendency for egg production to increase. Ten to twenty times as many rat units from viosterol as from cod-liver oil were necessary to induce approximately equal egg production, either number or total weight. The data on hatchability as influenced by addition of vitamin D were too erratic to be significant. The stimulating effect on egg production was secured with 2 per cent of cod-liver oil or 2 per cent of viosterol 20 D. Feeding 8 per cent of cod-liver oil definitely decreased egg production. Increasing the vitamin-D intake much above these figures caused decreased hatchability. Between 1 and 2 per cent of cod-liver oil is considered the optimum for laying hens, and 10 to 20 times the corresponding rat unitage, as viosterol, is employed.

Baird & Greene (39) studied the comparative vitamin-D requirements of growing chicks, turkeys, and pheasants. Substantiating

previous work they find that fifteen-day-old New Hampshire Red chicks grew better on their basal ration as the amount of cod-liver oil was increased up to 0.075 per cent. Chicks receiving 0.05 per cent cod-liver oil did not show leg weakness but were slightly rachitic at eight and twelve weeks of age as evidenced by low bone ash (39.1 and 39.7 per cent) of the tibias. Chicks receiving 0.075 per cent cod-liver oil were adequately protected to eight weeks but with sub-normal protection to twelve weeks. Using this last value as a basis of chick requirements, they found that the requirements of growing turkeys and pheasants, for the antirachitic factor, are one and one-half to three times those of growing chicks to twelve weeks of age.

St. John and his coworkers (40), using a cod-liver oil containing 270 I.U. of vitamin D per gram, have extended their studies on the vitamin-D requirements of chickens. For growth from one to sixteen weeks, pullets in confinement without sunshine required a minimum of about 17 I.U. of vitamin D per 100 grams of ration for satisfactory growth and calcification. From sixteen to twenty-four weeks the requirement was about 8 I.U. When sufficient vitamin D was afforded in the ration, additional vitamin from exposure to sunshine did not appear to further affect growth. Insufficient vitamin D apparently caused an increase in the number and severity of crooked keel bones.

Laying pullets had the ability to store vitamin D during growth, which appeared not to be exhausted until after three months' egg production.

Lack of sufficient vitamin D from either cod-liver oil or sunshine irradiation seriously retarded egg production. Inadequate vitamin D reduced egg size. Sixty-seven I.U. of vitamin D from cod-liver oil, per 100 gm. of ration, were found necessary for satisfactory egg production. Inferior shells were characteristic of inadequate vitamin D. Breeding hens kept out of sunlight and in confinement required 135 I.U. of vitamin D from cod-liver oil per 100 gm. of ration to produce satisfactory hatchability of eggs.

Titus and his coworkers (41) find that 0.5 per cent of viosterol, 16 D, in the diet of the chicken receiving graded amounts of cod-liver oil, had no apparent effect on the transfer of vitamin A to the egg. Titus and coworkers found the eggs from hens receiving no vitamin-A or -B supplement had about 20 units (Sherman-Munsell) of vitamin A per gram of yolk, while eggs from birds fed 8 per cent

of cod-liver oil had about 80 units. Those from pullets fed 1 to 2 per cent of cod-liver oil contained about 40 units.

Sherwood & Fraps (42) fed a ration of yellow corn, shorts, wheat bran, meat and bone meal, and oats, with and without alfalfa-leaf meal, to three groups of White Leghorn fowls. The fowls received 224, 336, and 444 Sherman-Munsell units of vitamin A, respectively, in the feed consumed daily, much of it being derived from 4 to 8 per cent of heat-dried alfalfa-leaf meal. The vitamin-A potencies of feed and eggs were estimated by rat assays. The mortality was highest (30 per cent) in the group receiving the smallest amount of the vitamin (no alfalfa). The fowls receiving 444 units of vitamin A were heaviest, followed by those receiving 336 units. These differences in weight appeared after the fourth month of the experiment. The birds receiving the lower amounts of vitamin A laid approximately the same number of eggs. Those which received 444 units daily laid 15 per cent more eggs than the other groups.

The vitamin-A content of the eggs of all lots decreased as the laying period progressed. The decline was greatest in the lot taking the largest intake of vitamin. At the close of the period of nine and one-half months, the egg yolks from the different lots contained 6, 12, and 15 units, respectively, per gram. In two hatching tests the percentage of eggs hatched increased with the increase in amounts of vitamin A in the feed, but the percentage hatched was not closely related to the amount of vitamin A in the eggs.

The units of vitamin-A potency required in the feed, in addition to maintenance, for one unit increase in the eggs, decreased as the average number of units fed increased: one unit in the eggs required 6.3 units in the feed when 270 units a day were fed, 5.7 units when 336 units were fed, and 4.0 units when 444 units were fed daily. The authors conclude that rations usually fed to laying hens do not supply enough vitamin A for maintenance and high egg production unless the hens have access to green grass or similar green feed. It seems probable that laying fowls which do not have access to green feed, and are fed many of the ordinary laying feeds, are likely to break down from deficiency of vitamin A during the second and third year, or sooner.

Koenig, Kramer & Payne (43) report data on the vitamin-A content of eggs as related to rate of production, from high producers, low producers, and hens receiving a pigment-poor ration. The ration for liberal vitamin A contained yellow corn and about 6 per cent

of alfalfa. The ration producing pale yolks contained white corn and no alfalfa.

Both high- and low-producing pullets, nearing the end of the first four months of production, laid eggs with yolks of similar vitamin-A content, at least 25 Sherman-Munsell units per gram. The period of production was too short to bring about marked differences in vitamin-A content of the eggs, but pullets of high- and low-production records laid eggs with dissimilar vitamin-A content near the close of the first year of egg production. Eggs from the low producers showed 33 units, and those from high producers showed 20 units of vitamin A per gram. The high producers had decreased and the low producers increased the vitamin-A content of their yolks as compared with the content of similar eggs produced at the end of four months of laying. Pale eggs produced on a ration devoid of carotene and xanthophyll but supplying vitamin A in the form of cod-liver oil contained 25 units of vitamin per gram.

Gillam & Heilbron (44) made a spectroscopic examination of egg-yolk fat obtained from hens receiving a diet mainly of maize. After saponification and the removal of sterols, the unsaponifiable matter was fractionated by the phase method. The carotene and xanthophyll fractions, thus obtained, were further analyzed by chromatographic methods. Vitamin A was obtained free from carotenoids. In quantity it varied from 0.05 to 0.10 mg. per 100 gm. of yolk. The chief pigment of the petroleum-phase fraction was cryptoxanthine (0.19 mg.) which was present in much greater amounts than carotene (0.015 mg.). The total carotenoid content and vitamin-A content of the yolk fat could be raised by feeding grass to the hens.

Towbin, Gorodissky & Drobona (45) report that operative trauma of one eye in rabbits caused increase in the glycolytic capacity and the total nitrogen content of the lens and aqueous humor, and in the sugar content of the aqueous humor but not of the lens. Insertion of carotene into the eye enhanced the increase of glycolytic capacity and of total nitrogen content, but partially prevented the rise in sugar content. Interference with one eye was not without effect on the other.

Vitamin A in the retina.—Wald (46) states that when visual purple is bleached by light in the eye, the visual yellow (retinene) is formed, the yellow color fades slowly, the retina becoming colorless. He believes that, at this point, retinene has disappeared, being trans-

formed entirely into vitamin A. In the living animal vitamin A is transformed into visual purple, thus completing the cycle. This cycle is not a perfect one, since some of the vitamin A is apparently lost in the process. The experiments were performed on frogs.

Vitamin-A requirements of very young puppies.—Frohring (47) has prepared a synthetic milk which is suitable for studies with very young animals, of vitamins A, B, G, and D. With this food he determined the vitamin-A requirements of growing puppies. This appears to be the first attempt to determine the minimum vitamin-A requirements of the dog. No growth response was obtained with puppies restricted to the synthetic milk at weaning (or at the latest one or two weeks later), when carotene was supplied in amounts corresponding to the curative dose per 100 gm. of body weight of the rat. Many puppies died on such dosages. With higher levels of carotene growth responses were obtained. The minimum curative dose which effected definite increase of weight was 20 U.S.P. units per 100 gm. of body weight per day. The weight used for these figures was the weight at the beginning of the curative dose.

With the daily minimum curative dose no blue units were found in any of the livers of animals killed at the end of the experiment. Even the control, getting 7,000 U.S.P. units daily from the beginning, showed a reserve of only 2 blue units per gram of liver. Loss of appetite was one of the first symptoms observed in vitamin-A depletion.

VITAMIN E

Ringsted (48) has studied the paresis which Evans & Burr (1928) and Mason (1933) observed in young rats, the progeny of mothers restricted to a diet very poor in vitamin E. Ringsted finds the clinical phenomena in these paretic rats similar to the neuropathic disturbances described by Aberle (49) in rats deprived of vitamin A to the point of bringing the weight increment to zero, or to the development of xerophthalmia, but the animals manifesting the vitamin-E syndrome differ from these in essential points.

In vitamin-E deficiency there is a sudden development of a spastic paralysis of the hind legs, feet, and toes in a period of pronounced growth, from the twentieth to the twenty-fifth days. A few that primarily show a relaxed paresis also end in a spastic condition. Ringsted's animals were almost adult when paresis set in and it was of the relaxed type. It developed slowly, so that it was possible to differen-

tiate various stages. It was accompanied by disturbance of sensibility affecting especially the deep-seated and to a less degree the cutaneous sensibility of the legs and tail.

Aberle described in her vitamin-A-deficient animals: spreading of the hind legs, when in motion increasing to pronounced straddling, ataxia, later on spasticity, and finally helpless trailing of the hind quarters and hind legs when walking. She did not observe disturbances of sensibility or atrophy of the hind quarters or hind legs, or atrophic disturbances of the skin or fur. She observed incontinency of urine which was not seen in vitamin-E-deficient rats of Ringsted. The latter noted in his parietic animals greatly distended bladders. The symptoms described by Aberle were cured by large doses of vitamin A, but those of the vitamin-E-deficient rats were not so curable. Ringsted emphasizes that the skin and nervous symptoms in vitamin-E-deficient rats do not resemble those seen in rats in experimental beri-beri or in rat pellagra. He believes the symptoms described to be characteristic of vitamin-E deficiency.

Barnum (50) observed considerable variation in the vitamin-E content of eggs, depending on the amount of the vitamin in the diet of the hen. Eggs produced on a normal diet were partially effective at a 3-cc., and fully effective at a 5-cc. daily level in the curative type of experiment with rats, whereas a 10-cc. level, daily, of eggs from a vitamin-E-deficient diet was insufficient. Thus the eggs from the normal diet were fully three times as potent in vitamin E as those from deficient diets. The eggs from the normal and the wheat-germ-supplemented diets showed the highest vitamin-E content, along with the lowest embryonic mortality during the first four days of incubation. Barnum believes that from the hatchability standpoint the richness of the diet in vitamin E and the ability of the hen to transmit this factor in an active form into the egg in sufficient amounts is a matter of considerable importance. Fifteen per cent of wheat germ supplementing the vitamin-E-deficient diet resulted in eggs high in vitamin E, but one head of lettuce per seven birds daily did not increase the vitamin-E content or markedly improve egg hatchability, although it reduced the first four-day mortality over that of the deficient diet.

The effect of vitamin-E deficiency on the hypophysis cerebri in the female rat.—Stein (51) has reviewed the literature on the *hypophysis cerebri* in rats as influenced by deficiency of vitamin E, and reports the findings of histological study of ninety-one glands from female rats. He found no significant differences in the weight of the

whole hypophysis or in the absolute and relative volume of the lobes in nineteen female rats made sterile by a deficiency of vitamin E, as compared with the lobes of the hypophysis from nineteen pregnant rats cured of vitamin-E deficiency. A differential count of the cells in the anterior lobe showed no significant difference in the percentage of the cured and non-cured. This is particularly true of the basophilic cells. No evidence of any histological change could be found resulting from vitamin-E deficiency such as has been claimed for male rats.

Stability of concentrates of vitamin E toward oxidizing and reducing agents.—Olcott (52) finds that vitamin E is destroyed by ozone, perbenzoic acid, potassium amide, potassium ethylate, and chlorine. Chlorinated and brominated concentrates may be reactivated by boiling with zinc and hydrochloric acid in methanol. Hydrogen bromide does not attack the vitamin. Cottonseed oil is as satisfactory as wheat-germ oil for the preparation of active concentrates. Concentrates of vitamin E exhibit a band in the ultraviolet absorption spectrum at 2940 Å, apparently not associated with the vitamin activity.

Evans & co-workers (53) describe methods for the preparation of vitamin-E concentrates. The wheat-germ oil used as raw material should be fairly fresh. After extraction the saponification of the oil should be carried out with methanol as solvent. Most fat solvents, other than ethyl ether, are unsuitable for extraction of the non-saponifiable fractions, unless considerable alcohol or similar liquid is also added. Wheat-germ oil, if sealed in vacuum in glass, appears to retain its vitamin-E content unimpaired for several years at room temperature.

Askew (54) studied a rich concentrate of vitamin E, and a crystalline substance derived from vitamin-E preparations, by the method of surface films. Both substances appear to have condensed polycyclic structures. The possibility that the crystalline substance is identical with β -amyrin is discussed.

Olcott (55) reports that vitamin E contains one or more hydroxyl groups and forms biologically active esters with acetic acid and benzoic acid. Its methyl and ethyl esters are inactive. The urethane derivative is inactive but alkaline hydrolysis regenerates the vitamin. Hydrogenation at high pressures does not destroy the vitamin. The activity of the esters suggests that vitamin E may occur in nature either free or esterified as do the sterols. The band at 2940 Å in the absorption spectrum of concentrates from wheat-germ and cottonseed

oils is not a property of the vitamin, but of a substance difficultly removable from it.

Drummond, Singer & Macwalter (56) separated from the non-saponifiable fraction of wheat-germ oil: sitosterol, dihydrositosterol, ergosterol, dihydroergosterol, lutein, kryptoxanthin, β -amyrin, squalene, a second hydrocarbon of the squalene type, a new hydrocarbon, probably $C_{18}H_{32}$, and a lipochrome which may be γ -carotene or rubixanthine. None of these substances appears to be related to the vitamin activity. Fractional adsorption on aluminum oxide was found to effect considerable concentration of vitamin-E activity. The most potent fraction obtained was effective in rat tests in doses of 0.1 mg. daily. Analytical data on highly active fractions of pale yellow, viscous oils suggest polycyclic structure. Two oxygen atoms are present in the molecule which has a molecular weight of about 440. An acetyl derivative was formed. Reactions with iodine and hydrogen suggest the presence of three reactive double bonds. Whether these observations relate to the vitamin or some associated substance is not certain.

The active fractions show absorption with a maximum at 294 $m\mu$ and a minimum at 267 $m\mu$. The vitamin activity tends to run parallel with the intensity of this band. The absorption at 294 $m\mu$ disappears on acetylation but reappears on hydrolysis.

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DEPARTMENT OF BIOCHEMISTRY
THE JOHNS HOPKINS UNIVERSITY
SCHOOL OF HYGIENE AND PUBLIC HEALTH
BALTIMORE, MARYLAND

NUTRITION*

By ARTHUR H. SMITH

*Department of Physiological Chemistry,
Yale University, New Haven, Connecticut*

During the year or more just passed the record of investigation in nutrition is characterized in part by the considerable attention devoted to the fundamental requirements of the chick. This species is of great commercial importance and therefore the relation of diet to growth (for meat production) and to egg production is of practical significance. However, a more recent interest attaches to the chick because of its extremely rapid growth and there is an increasing number of studies bearing on the fundamental food demands of this species in the light of the newer knowledge of nutrition. Thus, the requirements for vitamins, minerals, and proteins have continued to receive the attention of investigators.

It seems well established that the requirement for protein by the chick for optimal growth varies with age. St. John, Carver, Johnson & Brazie (1) fed a basal ration containing 10.9 per cent protein from plant sources and superimposed on this herring meal, so that final concentrations of 13, 17, and 21 per cent protein were obtained. During the first ten weeks of age, the most rapid growth was obtained with the rations higher in protein (17 per cent and 21 per cent); from the tenth to the twenty-fourth week only a relatively small advantage lay with the higher concentrations and at thirty-two weeks of age all groups weighed approximately the same. Liberal protein in the early weeks of life appeared to favor earlier egg production. The optimal concentration of protein in the ration of chicks to six weeks of age was about 25 per cent, according to McConachie, Graham & Branion (2); it decreased with age, after ten weeks being approximately 15 per cent; over the first twelve weeks' period the average concentration, optimal for growth, was 19 per cent. Roberts & Carrick (3) stated that if a ration containing a relatively high proportion of protein was fed for four weeks and then changed to a somewhat lower level of protein, the chicks were as heavy at twelve weeks as if they had been given the first ration for the entire time. However, this study loses some of its point because of the small differences in concentra-

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tion of protein between the various experimental diets. Ducks appear to utilize protein somewhat better than chicks; on a ration containing approximately 18 per cent protein of good quality, optimal growth (faster than that of chicks) was observed by Hamlyn, Branion & Cavers (4).

In a study of fish meals as a source of protein for chicks, Record, Bethke, Wilder & Kennard (5) found that when one-third to one-half of the meat scrap, in a diet containing this food material and skim milk, was substituted by fish meal, growth was enhanced. However, there was considerable variation in the nutritive efficiency when used as the only protein supplement in a grain-bran basal ration. A further investigation by Record, Bethke & Wilder (6) led to the conclusion that the method of manufacture of the fish meals is an important factor in determining their value as protein supplements in the rations of chicks. In an effort to formulate the nutritive value of protein concentrates of various qualities for the chick, Almquist, Stokstad & Halbrook (7) partitioned the total nitrogen by chemical precipitations and *in-vitro* digestion. Their protein-quality index is (percentage of total nitrogen precipitated by copper) — (percentage of total nitrogen not digestible + 0.6 per cent total nitrogen soluble in hot water) + (0.4 per cent total nitrogen precipitated with phosphotungstic acid). Good correlation was observed between this protein-quality index and the growth of chicks on their experimental rations.

In view of the possibilities in the use of chicks as experimental animals in nutrition studies, new efforts have been made to formulate simplified and, in some cases, purified rations. Using a basal ration of casein, dextrin, yeast, salts, and cod-liver oil, Kline, Elvehjem, Keenan & Hart (8) found that in addition to an antiparalytic factor (B_4), a growth factor found in the residue from water-extracted liver is required for growth of chicks. This substance added to a natural grain ration also stimulated development. Excellent growth and low mortality have been observed in chicks on the following simplified ration: crude casein 20, wheat-germ middlings, 10, yeast 15, corn starch 46, salt mixture 4, and cod-liver oil 5 [Van der Hoorn, Branion & Graham, Jr. (9)]. However, when the crude casein was purified with 0.1 per cent acetic acid, growth was retarded and "arthritis" ensued. These evidences of malnutrition disappeared when the ignited acetic acid extract of the crude casein was given. A report of the possibilities of chick nutrition with simplified rations was given by Hogan, Boucher & Kempster (10). Using the experimental diet which pre-

viously had proved reasonably satisfactory for growth of chicks to six weeks of age, four generations were reared. Egg production was fairly satisfactory but the fertility of both sexes was low. Considering the known deleterious effect of confinement of chicks in the laboratory, the Missouri investigators conclude that the ration used in these studies is distinctly promising.

In addition to the foregoing studies, there has been a number of observations on the requirement of chicks for the various vitamins. Some of the observations have an important bearing on our current conception of the biochemistry of these food factors. This phase of nutrition will doubtless be discussed in another section of the *Review*.¹

CARBOHYDRATES IN NUTRITION

Lactose has continued to elicit the interest of investigators. A comparison of the nutritive values of sucrose, glucose, and lactose was made by Koehler & Allen (11). Using adult rats which had lost 40 to 50 gm. body weight in the course of a period of restricted feeding, the appropriate sugar was mixed with the restricted allowance of food to bring the total caloric value to the maintenance level. The gain in body weight, thus permitted, was a function of the nutritive value of the sugar. In the course of thirty-two days' re-alimentation, the gain in weight on lactose was only 64 per cent of that when glucose was given and 61 per cent of the gain with sucrose. The changes brought about by intestinal bacteria probably accounted for the loss of some of the lactose to the animal. Whittier, Cary & Ellis (12) used the paired-feeding method in comparing lactose, sucrose, and dextrin on the basis of growth of young rats and longevity. Concentrations of the sugars as high as 63.5 per cent were fed. More rapid growth was observed with lactose than with sucrose and at this stage of development the increase in weight was not due to fat. Lactose likewise definitely favored longevity in these studies. Dextrin paralleled sucrose rather than lactose in its effects, a circumstance which indicates that the favorable influence of lactose does not depend on the stimulation of the growth of aciduric bacteria in the intestine.

Along with the other observations on the nutritive value of lactose are three reports connecting this sugar and one of its hydrolytic products with ocular pathology. In two-thirds of the rats fed a purified diet containing 70 per cent of lactose, Mitchell & Dodge (13) observed

¹ Cf. chapters on vitamins. (EDITOR.)

bilateral cataracts. This change occurred in a period of ten weeks on this level of lactose and required somewhat longer when less of the sugar was fed. Starch, maltose, dextrin, and sucrose, given in similar concentration, produced no obvious abnormality. This work was extended by Mitchell (14) by feeding galactose at both 35 per cent and 25 per cent levels in an otherwise adequate purified ration. In less than three weeks mature bilateral cataract had developed. Inasmuch as 35 per cent of galactose is the equivalent of 70 per cent of lactose, it appears that the former sugar is largely responsible for the observed abnormality under these conditions. Furthermore, there was a localization of calcium in the eyes with cataract, which fact led to the conclusion that the sugars involved caused a disturbance of mineral metabolism. Yudkin & Arnold (15) produced cataracts in the eyes of both weanling and adult rats by feeding a diet containing 50 per cent of galactose. The sugar was thought to interfere with the metabolism of the lens, the changes occurring at the sites of growth of the tissue.

The rate of formation of glycogen in the liver may be used to evaluate the nutritive value of sugars. Feyder & Pierce (16) studied the comparative efficiencies of sucrose, glucose, and corn syrup in glycogenesis in the rat. Glucose and sucrose showed approximately the same rate of glycogen formation during the first two hours after administering the sugars. At the end of the third hour, however, the rate with glucose diminished whereas that observed after sucrose feeding continued as before. The maintenance of the rate of glycogen formation with sucrose was later shown to be due to the presence of levulose in the hydrolytic mixture. Sucrose and glucose also differ in the extent to which they promote the formation of fat, according to Feyder (17). On an adequate purified diet containing 68 per cent of carbohydrate, the results of paired feeding showed uniformly that sucrose promoted more rapid gain in weight and that the gains were due largely to fat. The two foregoing studies place new emphasis on the nutritive value of levulose.

Further investigations on the nutritive value of bread have been carried out. Laug, Garavelli & Nash (18) studied the utilization of the non-cellulose carbohydrates of white, whole wheat, and rye breads using as criteria the excretion of reducing substances in the urine of both phlorhizinized and normal dogs. It appeared that the carbohydrates of whole wheat bread are absorbed more rapidly than are those of white or rye bread and that the carbohydrates of rye bread are utilized less completely than are those of the other breads. In a further

study of the carbohydrates of bread, Eyerly, Oclassen & Killian (19) gave bread, glucose, invert sugar, and sucrose to normal human male subjects after fasting. Under these experimental conditions the carbohydrates of bread produced minimal increases in blood sugar and in sugar in the urine.

Although the soy bean is a very important source of food in the Far East, its use as a constituent of the diet in one form or other is increasing elsewhere, particularly in infant nutrition (20). The protein of soy beans has received considerable attention but little is known of the biological value of the carbohydrate. In a study on rats, Adolph & Kao (21) compared the utilization of starch, soy-bean carbohydrate, and cellulose. The *in-vitro* digestion, the formation of liver glycogen, the respiratory quotient, and extra sugar eliminated after phlorhizin administration were used as criteria for judging the relative nutritive value. About 27 per cent of the total carbohydrate of soy bean is digested by diastase; the soy-bean carbohydrate forms liver glycogen almost as readily as does starch; the respiratory quotient is somewhat lower after ingesting soy-bean carbohydrate than after starch and it has a utilization coefficient roughly one-third that of starch.

A study of the behavior of carbohydrates in maternal and fetal tissues which has significant implications in nutrition has been reported by Corey (22). The glycogen in the maternal and fetal liver, in the placenta, and in the fetal muscle was determined in rats under various experimental conditions. Adrenalectomy of the mother produced a decrease in the glycogen of both maternal and fetal livers. Injection of insulin had a similar effect. The glycogen content of both livers was increased by the injection of glucose into the mother as was the glycogen of the fetal liver. These experimental procedures had no influence on the placental glycogen.

The absorption, assimilation, and utilization of carbohydrates are considered by Pierce (23) in an editorial review. Included in this consideration are also the combustion and specific dynamic action of this group of food substances as well as their transformation to glycogen and fat in the body.

The relation of dental caries to nutrition continues to receive attention. Bunting (24) has concluded that inherent (inherited) characteristics of the structure are of more significance in the resistance to caries than are ordinary dietary adjustments. That carbohydrate plays a rôle is implied by Bunting's advocacy of an adequate diet low in sugar as an aid in resistance. The suggestion is made further that diet is

important in this connection rather in the control of the environment of the tooth than in change of resistance of the tooth itself. On the other hand, Day, Daggs & Sedwick (25) fed to rats adhesive rations containing as much as 55 per cent of sugar for relatively long periods without observing an increase in the incidence of caries. Additional factors are involved, according to Rosebury, Karshan & Foley (26). They believe that the physical effect of impaction of large fermentable food particles into the fissures of molars together with a deficiency of minerals and vitamins are important in the etiology of dental caries in rats.

In a series of metabolic experiments on seventeen girls from seven to eleven years of age in connection with a dental study, Hubbell & Koehne (27) added sugar to the adequate basal diet. An increase of 6 per cent in the calories as sugar had no effect but when the energy value was raised 18 per cent by sugar there followed an increase in retention of nitrogen and of phosphorus with a decrease in calcium. The calcium was lost in the urine rather than in the feces. In a survey of extraordinary scope, Jones (28) discusses the changing concept of the adequacy of the diet in relation to dental disease. The conviction is set forth that fruits and vegetables are definitely protective, the potential alkalinity of these dietary constituents being stressed as a factor in the control of dental disease.

PROTEINS IN NUTRITION

Considerable attention has been given to the effect of heat and other forms of radiant energy applied to protein upon the nutritive value of that protein. Casein heated in thin layers at 140° C. for one-half hour was distinctly altered according to Greaves & Morgan (29). Growth in young rats was better when raw casein was the protein of the diet than when heated casein was used. Heated casein at levels of 12 per cent and 15 per cent was effectively supplemented by histidine and lysine. Determinations of the biological value showed a definite difference between the two proteins, raw casein showing a value of 68 and heated casein, 58. Morgan & Kern (30) extended the scope of the foregoing investigation by using meat: raw, boiled at ordinary pressure, heated for seven minutes at 15 pounds pressure, and for one hour at 15 pounds pressure. The biological value of the raw meat determined with rats was 67, and that of the meat heated for one hour at 15 pounds pressure was 56; the other heat-treated meats showed values between the two. Growth of young rats again was least satisfactory on

diets, the protein of which was provided by the heated meats. That there is a difference between proteins as regards heat injury is evident from the report of Chick, Boas-Fixsen, Hutchinson & Jackson (31). Using the maintenance of nitrogen balance in adult rats as a criterion, it was concluded that the biological values of lactalbumin and casein were not lowered by heating for seventy-two hours at 120° C., whereas the value for casein was decreased from 64 to 44 by heating for forty-four hours at 150° C. The digestibility of both proteins was diminished by heating.

The close association between digestibility and biological value for liver protein is emphasized by the observation of Seegers & Mattill (32) that heating beef liver for seventy-two hours at 120° C. or extraction with hot alcohol for one hundred and thirty hours markedly diminished the biological value (nitrogen-balance method). It was also pointed out that water seems to be required to bring about the change in the protein by heat, a change not involving any alteration in the amino acids themselves. A somewhat similar conclusion has been reached by Bickel (33) on the basis of his observations with casein irradiated by mesothorium. In contrast to the non-irradiated protein, it caused a decrease in the weight of male rats and gave chemical evidence of poorer biological value. The influence of drying temperature on the nutritive value of fish-meal protein was examined by Wilder, Bethke & Record (34) using the nitrogen-balance method with rats. There were decreases in digestibility and biological value with increase in drying temperature of haddock meal. As might be expected the protein from the edible portion, i.e., trimmings from filets, was more digestible and better utilized than was that from heads and tails.

The interrelation between the level of protein in the diet and the utilization of both protein and the other dietary constituents has been studied. Chick, Boas-Fixsen, Hutchinson & Jackson (31) examined the biological value of the protein of various grains, milk, lactalbumin, and casein and showed that, with the exception of whole milk protein, the biological values decreased as the concentration of protein in the diet increased. The amounts of protein in the diets ranged from 3 per cent to 10 per cent. Other components of the ration were not efficiently utilized by hogs if there was insufficient protein, according to Landis & Burckhardt (35). For rapid fattening 140 to 150 gm. of protein per day are necessary up to 20 kg. body weight, whereas for weights of 70 to 80 kg., as much as 280 to 300 gm. of protein are required. Forbes, Swift, Black & Kahlenberg (36) fed casein at 10, 15, 20, and 25 per

cent levels, in purified diets, to rats. As the proportion of protein increased, there was greater increase in body weight, the gains were made at a progressively smaller energy cost, the digestion and retention of protein were more efficient although the rate of increase in body protein diminished and the rate of loss of nitrogen in the urine increased. Under the conditions of this experiment there was no regular change in the amount of fat laid down, although in most cases the proportion of fat to protein stored, diminished. Another evidence of the mutual dependence of foodstuffs for satisfactory utilization is the observation of Swift, Kahlenberg & Forbes (37) that a deficiency of cystine in a diet otherwise comparable to the control ration results in a disturbance of utilization of energy-producing nutrients. Depression of growth, appetite, and storage of energy and nitrogen occurred.

Considerable significance attaches to the method of measuring the biological value of proteins. It has been pointed out by Schneider (38) that one component of the metabolic nitrogen in Mitchell's method varies with the intake of the nitrogen-free diet. It becomes essential, therefore, to assure the consumption of more than a certain minimum of the nitrogen-poor food. Mitchell (39) has given details of his observations on the same subject and comes to the same conclusion. Ashworth (40) has called attention to the influence of the nature of the protein fed prior to the period of determining the endogenous nitrogen excretion. If the period is relatively short this influence is not operative but with long periods, with resulting depletion of body stores of protein, it assumes importance. Boas-Fixsen (41) has reviewed the topic of biological value of proteins in nutrition. The various methods which have been employed are surveyed, the results tabulated, and the evaluation of the biological value of proteins for various functions discussed.

Contributions have been made indicating that it is difficult, if at all possible, to influence the amino acid composition of body proteins by adjusting the proteins in the diet. Calvery & Titus (42) fed to groups of hens rations which contained in one case all the protein as wheat middlings, in another as corn and corn gluten, and in a third as soy-bean press cake. The albumin and vitellin from representative eggs were crystallized and these, together with the whites and yolks, analyzed for various forms of nitrogen, sulfur, and for certain amino acids. In no case was there a marked difference in composition of the egg proteins which could be correlated with the food protein. In a study with rats, subjected to underfeeding as well as to restriction of cystine and re-

alimentation, Lee & Lewis (43) demonstrated that the compositions of proteins from muscle, liver, and kidney were unchanged by the alterations in the experimental diets. Nitrogen, total sulfur, tyrosine, cystine, and tryptophane were determined in the tissue proteins.

The utilization and biological value of the protein in a variety of foods have been determined with human subjects and with experimental animals. French & Mattill (44) compared the protein of whole wheat, rye, and white bread, the latter supplemented with milk. The protein of rye bread was not utilized as well by young rats as were the proteins from the other two samples, and it was found that all the proteins were better utilized by adult animals than by young ones. Using the nitrogen-balance method and feeding the bread proteins at a level of 5 per cent, it was found that in general the biological value is in the same class with meat, inferior only to milk and egg proteins.

The vigorous search for additional essential amino acids has been continued by W. C. Rose and his coworkers. They had previously reported failure to obtain growth in young rats on diets, the nitrogen of which was supplied by a mixture of pure amino acids and glucosamine hydrochloride, but which were otherwise adequate. However, when casein, hydrolyzed casein, or the monoamino acid fraction (butyl-alcohol-soluble portion) was added to this diet, growth was promoted. Caldwell & Rose (45) demonstrated that the unknown growth essential is associated with the group of monoamino acids, the carbamates of which are insoluble in very cold water. They (46) showed, further, that the copper salt of this factor is readily soluble in water but only slightly soluble in methyl alcohol. There was every indication that it was an integral part of the casein molecule and that tryptic digestion of the casein produced no more of it than did acid hydrolysis.

Re-extraction of the later butyl alcohol extracts has resulted in a separation of the unknown growth essential into two components (47), neither of which alone will supplement the basal diet. One of these is soluble in butyl alcohol and has been identified as isoleucine. The basal diet contained isoleucine but not in sufficient quantities for growth. The second component of the unknown growth essential was isolated from hydrolyzed fibrin by McCoy, Meyer & Rose (48). A careful purification produced crystals, the chemical character of which was thoroughly investigated. This new essential amino acid is one of the four optically-active isomeric α -amino β -hydroxy butyric acids.

In an earlier contribution Womack & Rose (49) reported that by manipulation of the components of the amino acid mixture in the

basal ration, evidence was obtained that leucine is indispensable for nutritive well-being. The same investigators (50) also showed by a similar procedure that phenylalanine must also be considered to be essential. As a result of this extremely fruitful series of recent studies on the effect of feeding amino acids, four additional amino acids have been shown to be necessary for satisfactory nutrition: leucine, phenylalanine, isoleucine, and α -amino β -hydroxy butyric acid.

The nutritional deficiencies of gelatin have been studied by Kruse, Day & McCollum (51). The basal diet containing gelatin, tyrosine, tryptophane, and cystine was supplemented in one case with histidine and in another case with the monoamino acid fraction from hydrolyzed casein. The best growth under these conditions was obtained with 0.45 per cent of the former and with 5 per cent of the latter supplement. Gelatin, zein, and casein were compared by Mason & Palmer (52); using the nitrogen-balance method, retentions were 23, 57, and 74, respectively. Although casein and gelatin showed similar and high digestibility, that of zein was poor and variable. Haag (53) showed that the nutritive value of wheat-bran proteins was superior to those of alfalfa leaves, a difference not due to differences in apparent digestibility. Rats in groups of three, rather than in pairs, were used, the third rat receiving a mixture of the two proteins.

An evaluation of the nutritive value of soy-bean protein is of great practical importance, particularly in China and Japan where this legume is depended upon to a considerable extent for human food. Studies by Liu & Chen (54) indicate that the protein of soy-bean cake compared favorably in nutritive value with that of dried beef, pork, casein, and defatted beef muscle when all were fed at a level of 10 per cent. The proteins of soy-bean milk are more slowly hydrolyzed by trypsin than are those of cow's raw milk, whereas with pepsin the soy-bean milk proteins are more rapidly digested, according to Adolph & Wang (55). Both enzymes together bring about a somewhat more complete digestion of cow's milk than of soy-bean milk.

The physical character of the diet may exert a detectable influence on the utilization of nutrients. Garrigus & Mitchell (56) reported that the value of corn as a source of energy for pigs weighing 135 to 155 lbs. was increased 3.5 per cent by grinding. It appears that this treatment had little effect on the value of corn as a source of protein. That bulk alone does not interfere with the digestibility of protein, however, was shown by Adolph & Wu (57) in experiments

on both rats and men. When the roughage employed caused a more rapid passage of food material through the intestine, however, digestibility values were lower.

On the basis of nitrogen- and phosphorus-balance studies, Long & Pittman (58) concluded that proteins from beef muscle and from liver are equally well utilized. On the other hand, when beef heart was compared with beef round, Kunerth, Chitwood & Pittman (59) observed a somewhat better utilization of protein from the cardiac muscle.

An extensive investigation of the economy of nitrogen during pregnancy has been carried out by Coons & Marshall (60) on six subjects. On the basis of observations during twenty-three balance experiments each covering a four-day period and one period of four weeks, it was concluded that the average nitrogen retention during pregnancy, under the conditions of life in Oklahoma, was 1.97 gm. per day. Hunscher, Hummell, Erickson & Macy (61) have described a striking example of the material gain to the maternal organism resulting from a completed reproductive cycle. The subject had been under more or less close experimental observation for eight years but the present study concerned the last 145 days of gestation, parturition, the puerperium, and the first eight weeks of lactation. During the last 145 days of gestation there were 377 gm. of nitrogen stored by the mother; during delivery, loss of body fluid and fetal adnexa accounted for 55 gm. of nitrogen; 45 gm. of nitrogen were lost during the lying-in period and 38 gm. in the milk during the last forty-three days of observation. There was thus a net gain to the maternal organism of approximately 250 gm. nitrogen from 145 days prior to parturition to 53 days postpartum.

A significant study of the protein requirement of pre-school children was made by Daniels and her associates (62). One hundred and twenty-one balances on twenty-four children showed that when intake of nitrogen and retention are based on milligrams of urinary creatinine, the daily requirement of protein is 3.2 gm. per kg. body weight or 1.5 gm. per pound when at least one-half of the protein is from animal sources.

FATS IN NUTRITION

The success attending the use of simplified experimental rations in elucidating some of the fundamental principles in nutrition has led to the attempt to simplify the fat component of such rations. Nutri-

tive differences between various esters of the same naturally-occurring fatty acid have been noted. Lepkovsky, Ouer & Evans (63) fed the free fatty acids of lard as a component of a purified diet; growth was satisfactory at a concentration of 25 per cent but not at 60 per cent, although at both levels the glycerides were adequate. The methyl and ethyl esters, although utilized at a level of 25 per cent, failed to promote satisfactory growth at a concentration of 60 per cent. Ethylene glycol, diethylene glycol, and propylene glycol were esterified with the fatty acids of lard; at a level of 60 per cent, only the last of these esters promoted growth in young rats.

The importance of bile in promoting the utilization of fat has been emphasized again by Riegel, Elsom & Ravdin (64) who studied the absorption of oleic acid from intestinal loops in dogs. Little, if any, was absorbed when the fatty acid alone was introduced. On the other hand, although absorption was facilitated by the addition of either bladder or hepatic bile, still better absorption was observed when sodium taurocholate was added to the oleic acid.

Sperry & Stoyanoff (65) used the paired-feeding method in order to determine the influence on growth and food utilization of relatively long-continued feeding of cholesterol; about 2 per cent of the purified sterol was included in a purified diet. The rats without added cholesterol grew better than the others, consumed more food, and showed a somewhat greater index of efficiency in the utilization of food. All the cholesterol fractions of the tissues of the cholesterol-fed rats increased, but the change was particularly striking in the cholesterol esters of the liver. The same investigators (66) added commercial lecithin, egg yolk, and calf brain separately to the diets of various groups of chicks. The diet with the largest concentration of cholesterol (calf brain) promoted an increase in liver cholesterol, as was observed with rats, but in the case of the chick there was a greater proportion of the cholesterol deposited in the free form.

The influence of corn, oats, wheat, and barley on the quality of body fat deposited in young growing rats was investigated by Olcott, Anderson & Mendel (67). Although the grains furnished 82 per cent of the total calories of the food, differences in fat content of the grains resulted in the following contributions to the energy intake by the respective oils: corn, 8.0 per cent; oats, 10.0 per cent; wheat, 3.0 per cent; and barley, 4.0 per cent. The iodine numbers of the body fat of rats fed corn and wheat were slightly higher than those of the other groups.

GENERAL

The history of investigation in nutrition indicates that edible fungi have long been looked upon favorably as food materials. The rather high content of total nitrogen led to early studies of the value of these plants as sources of protein. Recently Gorcica, Peterson & Steenbock (68, 69) examined *Aspergillus sydowii* for its content of factors indispensable for normal nutrition. The mould apparently contains a considerable amount of vitamin G and less, though effective quantities, of vitamins B₁ and B₄. The protein of this mould is of poor nutritive quality. Whole wheat and corn-gluten feed effectively supplemented the mould protein as did casein, skimmed milk, egg white, and yeast, but 0.5 per cent of cystine, 0.5 per cent of histidine, 1.0 per cent of tyrosine, or a combination of 0.5 per cent of cystine and 1.0 per cent of tyrosine, added to a ration containing 18 per cent of mould protein, showed little improvement in the growth of rats.

Quackenbusch, Peterson & Steenbock (70) extended these studies to one of the mushrooms commonly used for human food, *Agaricus campestris*. This variety proved to contain vitamins B and G but lacked some additional factor required for growth, which factor is contained in liver, autoclaved yeast, and alfalfa. A purified ration containing 1.0 per cent of mushroom was not toxic and promoted growth comparable to that of animals consuming equalized quantities of a good stock ration, but nutritive failure resulted owing to ultimate inability to maintain an adequate food consumption.

Wu & Wan (71) have continued their efforts to make a superior diet for experimental animals used for nutrition studies. They reported improved performance over a satisfactory milk whole-wheat ration when the following diet was given: whole wheat, 46; millet, 2.0; roasted soy bean, 10; dried beef, 10; cod-liver oil, 5; yeast, 5; salt mixture (McCullum), 4; and green vegetables twice a week. Thus far they have not been successful in constructing a vegetarian diet equal to this mixed ration. The use of so-called synthetic diets has been extended to guinea pigs, rabbits, goats, and sheep by Madsen and co-workers (72). The experimental rations consisted of casein, starch, sucrose, lard or oil, regenerated cellulose, and salt mixture. In some of the experiments cod-liver oil was included in the ration; in others, it was fed separately. In still other trials, a cod-liver-oil concentrate was substituted for the cod-liver oil. Goats were reared from weaning to 580 days of age and sheep to 480 days of age on these rations. The development, as shown by necropsy examina-

tion, was very nearly normal. The efforts to grow rabbits and guinea pigs on this type of diet were less successful, eventual failure being due to degeneration of skeletal muscles and resulting paralysis. Injury to heart muscle in the goats, sheep, and guinea pigs was associated by the authors with the feeding of cod-liver oil, although there was some injury even when all the cod-liver oil was omitted from the purified rations. Frohring (73) has described an artificial milk suitable for rearing puppies. It consists of vitamin-A-free casein, hydrogenated fat (Crisco), sucrose, salt mixture (Osborne & Mendel), linoleic acid, and water. It is supplemented with yeast, some of which was irradiated, and carotene. According to the report, puppies have been fed on this "milk" alone for two to three months; when supplemented with boiled rice and yeast, the "milk" promoted growth for eight months.

According to Blumberg (74) nutritive failure was observed on a highly purified ration consisting of fat-free casein, sucrose, salts, fat-free yeast, carotene in ethyl laurate, irradiated ergosterol, and ethyl linoleate. Wheat germ, wheat-germ oil, or egg yolk served as effective supplements; growth was resumed and muscular vigor and the oestrous cycle were restored.

The question as to the effect of pasteurization on the nutritive value of milk has received renewed attention. Using young rats and mineralized milk, both raw and pasteurized, Elvehjem, Hart, Jackson & Weckel (75) came to the conclusion that season and the quality of food eaten by the cow had more effect on the nutritive value of the milk than did pasteurization. According to them, milk of high nutritive value was not altered by the heat treatment but milk of poor quality may be affected. The current interest in the importance of curd tension of milk in nutrition gives weight to the observation of Espe & Cannon (76) that the content of fat in the milk influences the nature of the curd. Using calves with a fistula in the rumen, they found that milk with 3 per cent or 6 per cent of fat formed a friable curd whereas that from skimmed milk formed a curd that was viscous and stringy. Furthermore the fatty curd left the stomach sooner than did the skimmed milk curd.

The part played by roughage in promoting a feeling of comfortable fullness was touched upon by Harrop (77) in a report of success attending the use of a diet consisting of bananas and skimmed milk in the treatment of obesity. According to this plan six large ripe bananas yielding 569 calories and one quart of skimmed milk yielding

371 calories constitutes the sole diet until the desired reduction of weight is secured, or this regimen is alternated with the ordinary diet at intervals of two weeks. In six subjects nitrogen equilibrium was attained within two weeks on this regimen. That rats and mice can thrive on adequate rations rich in indigestible material has been shown by McCay, Ku, Woodward & Sehgal (78). As much as 20 per cent of regenerated cellulose was added to a purified ration and, in some cases, growth at a normal rate and prolongation of the life span were noted. The rat was found to digest more of the fiber from beet pulp than from bran or regenerated cellulose. Attention was called to the fact that the feces of the animals on these diets had a greater dry weight than could be accounted for by the undigested roughage.

The literature on changing rates of growth with change in nutrition has been extended by Mendel & Hubbell (79). Careful records kept for the past twenty-five years indicate that the growth rate of the rats in the colony of the Connecticut Agricultural Experimental Station has gradually increased with a marked change since 1931. This improvement in rate of development was accompanied by superior reproductive performance. The average body weight of males at twenty-one days of age is now 48 gm. and the average daily gain from twenty-one days to one hundred days of age is 4.0 gm. That a rapid rate of growth is not conducive to prolongation of life in the rat has been suggested by McCay, Crowell & Maynard (80). The mean age of the retarded-growth males was much greater than that of the rapid-growth males; this difference was not demonstrated in the females. One group of animals was restricted in its food intake for 766 days and another group for 911 days, after which food was given *ad libitum*. At these extreme ages, the capacity to grow was still evident though the final body weight attained was less than that of rats which reached a mature weight at a younger age. On the other hand, Sherman & Campbell (81) reported that the prospects of a long life were equally good for two groups of albino rats, one of which consumed an adequate diet and the other, a ration somewhat better when judged by rate of growth. It appears from this statistical study that the rate of growth and length of life vary independently of each other.

Further studies have been reported which relate diet to body structure and size of organs. Callow (82) has summarized some relationships which seem to be of practical significance. With reference

to hogs, it appears that the longer the body the greater will be the proportion of muscle to fat and the faster the animal will grow. Older animals possess a greater proportion of fat and the fat is firmer. The relation of iodine number of the food fats to the unsaturation of carcass fat is also stressed. Jackson (83) has summarized the effect of long-continued suppression of growth due to restriction to a "protein-deficient diet." Young rats were maintained for fifteen weeks on this ration, then re-alimented with a normal stock ration. After one year on the experiment the females had slightly exceeded the controls in body weight but the males weighed less than the controls. The test males had shorter bodies and tails and the head, skeleton, musculature, and eyeballs weighed less than these parts of the control rats. The females also had bones and eyeballs of lower weight than those of controls. It was suggested that the skeleton and eyeballs are particularly susceptible to injury by the early lack of protein.

The relatively large factor of safety ordinarily in evidence throughout the body is shown to exist with reference to digestion by the study of Handelsman, Golden & Pratt (84). The pancreatic juice was excluded from the intestine by ligating the ducts in the dog. Mixed diets were used and it was found that large amounts of nutrients were absorbed at the same time that considerable portions of the ingested fat and protein were lost in the feces. The data are exceedingly variable but in general it appears that the interference with the normal flow of pancreatic juice had little effect on the absorption of carbohydrate whereas the absorption of protein and fat was distinctly subnormal.

NUTRITION IN RELATION TO REPRODUCTION AND LACTATION

During the period under review, ingenious procedures have been devised to estimate the quantity and quality of the milk of the laboratory rat and the influence of various dietary adjustments upon lactation in this species. Assuming that the young rat consumes no other food than mother's milk for the first seventeen days of life, Daggs (85) has devised a lactation index. When the logarithms of litter weights are plotted from the fourth to the seventeenth day of life against the time in days, two straight lines are obtained which cross at the tenth day. By adding together the two growth constants derived from the plotted data a value is obtained which indicates the efficiency of lactation as reflected in the body weight of the litter. Daggs & Tombouliau (86) studied the lactation-promoting effect of

various glandular tissues, eggs, muscle, proteins, and amino acids, by the foregoing method, the materials providing the protein of an experimental ration not otherwise described. All the foregoing sources of protein showed the presence of a factor promoting lactation, but cystine alone or in combination with glycine, glutamic acid, and casein showed a striking effect, suggesting to the authors that cystine or glutathione acts as a mammary gland stimulant.

The relationship of the food material to the synthesis of the characteristic constituents of milk has continued to be a topic of investigation. Allen (87) studied the effect on milk-fat production brought about by adding fats as such to the rations of two groups of dairy cows, alike in breed, production, size, and nutritive condition. The basal ration consisted of combinations of ensilage, alfalfa, and mixed grains; for the experimental periods (six days long) the fat was melted and thoroughly mixed with the grain. Butter fat, tallow, lard, cottonseed oil, corn oil, linseed oil, soy-bean oil, peanut oil, and cocoanut oil were investigated. The fats promoted insignificant changes in volume of milk but all of the fats caused an increase in the milk fat. This was an effect of the fats themselves and not of the enhanced caloric value of the ration. In experiments in which 0.25 lb. to 1.25 lb. daily of the fat was fed, it was shown that the effect on the milk fat was proportional to the concentration of fat in the diet. On the other hand, using longer experimental periods (twelve and thirteen weeks), Maynard, McCay, Williams & Madsen (88) concluded that, although the differences between the effects of a high-fat and those of a low-fat ration in promoting an increase in butter-fat yield were generally in favor of the high-fat diet, they were too small to be considered significant. The fat content of the experimental rations was adjusted by suitable selection of the components of the grain mixture, and ensilage and hay were used as the sources of roughage. McCay & Maynard (89), in studying further the effect of feeding cod-liver oil to milch cows, came to the conclusion that the resulting decrease in the percentage of milk fat appears to be caused by the fatty acids of the oil rather than by the non-saponifiable part. Neither salmon oil nor shark-liver oil brought about this change consistently. Comparable analyses of blood from the jugular vein and the mammary vein showed that the total lipid was less in the blood leaving the mammary gland than in the jugular blood. Furthermore, determinations of the lipid phosphorus and the inorganic phosphorus did not support Meigs' theory

of the origin of milk fat. However, Maynard & McCay (90) showed that the character of the food fat is reflected in that of the milk fat. Using goats and cows, they fed fats with various degrees of unsaturation; the iodine number of the milk fat changed in a parallel way within twenty-four to forty-eight hours. This change was not reflected in the lipids of the blood, which fact indicates that the mammary gland exerted either a highly selective action or transformed the fat in the gland itself.

The importance of blood glucose in the synthesis of lactose by the mammary gland was emphasized by *in-vitro* studies of Grant (91). Slices of active gland from guinea pigs were suspended in oxygenated physiological salt solution for six hours. The tissue was removed, ground in sand, the suspension deproteinized, and lactose determined in the filtrate. Glucose, fructose, mannose, and galactose were examined. Glucose was almost completely converted to lactose by the tissue whereas only a small amount of the disaccharide was formed from the other sugars. Tolstoi (92) determined the concentration of lactose in the milk of diabetic female subjects over a period of hours during which the blood sugar was raised by the ingestion of glucose. In another group, the glucose concentration of the blood was decreased by insulin. In neither group was the proportion of lactose in the breast milk altered significantly, a fact which demonstrates the essential constancy in the quality of the milk of a given species.

That the newer conceptions regarding the indispensability of certain amino acids in nutrition has influenced practice in animal husbandry, is indicated by the proposal of Fowler, Morris & Wright (93). They fed rations poor in lysine to milch cows and observed decreases in the quantity of milk produced as well as increased loss of nitrogen in the urine, whereas high yields of milk protein ordinarily are accompanied by diminished loss of nitrogen in the urine. This amino acid thus becomes a limiting factor in the nutritive value of rations for milk production and the authors suggest that protein-feeding standards for milk production be revised to take cognizance of the quality of the concentrate employed. Hughes & Hart (94) have studied the milk production of two sows and have determined the chemical composition of twenty-three samples of colostrum and fifty samples of normal milk from the sow. The average composition of the normal milk is as follows: solids, 17.98 per cent; fat, 6.8 per cent; protein, 6.2 per cent; ash, 0.97 per cent; calcium, 0.25 per

cent; and phosphorus, 0.15 per cent. As might be expected the total solids and protein of the colostrum were elevated, the average values for the former being 28.0 per cent and for the latter 15.5 per cent. The paper gives a good bibliography on the comparative composition of the milk of various species. In view of the wide use of the rat in nutrition studies, the publication by Mayer (95) of the composition of the milk of this species is of particular interest. The analyses were made on the stomach contents of suckling rats ten to eighteen days of age. They were kept from the mother for twelve hours, allowed to suckle, and then killed and the analyses made on the stomach contents. According to the author, there was no evidence of digestion at the time of analysis. The weight of the stomach contents increased rapidly with age. As might be expected there was a wide variation in proportion of solids in the coagulated milk removed from the stomachs. A typical analysis showed the following: water, 77.8 per cent; protein, 6.9 per cent; carbohydrate, 3.4 per cent; fat, 12.4 per cent.

Coons, Schiefelbusch, Marshall & Coons (96) have reported an extensive series of metabolism experiments on six human subjects. Balance studies in twenty-five experimental periods were made on calcium, phosphorus, magnesium, sodium, potassium, sulfur, chlorine, iron, and nitrogen. The diets were selected by the patients who lived at home. Contrary to many reports in the literature, no negative calcium balances were observed; on diets not noticeably superior from the point of view of calcium content, the average storage was 1.40 gm. daily or 2 or 3 times the estimated needs of the fetus. Positive balances for phosphorus and for magnesium were likewise observed. In each of twenty experimental periods an excess of base ranging from 59 to 770 cc. of 0.1 *N* base was retained. Although no negative balances for iron were observed, most of the subjects failed to retain enough of this element to satisfy the calculated needs of the developing fetus. The average daily nitrogen retention was between 1 and 2 grams. It is noted that the storage of both iron and nitrogen decreased as pregnancy proceeded.

The deleterious influence of undernutrition on sex physiology has been indicated. Adsell & Crowell (97) studied two groups of young rats, one containing animals 40 gm. in weight, the other with rats 80 gm. in weight. The rats were so restricted in food-energy intake that growth was retarded. The age of opening of the vagina increased with the severity of the stunting; it appears that age is more impor-

tant than body weight in determining the opening. The evidence indicates that the requirements of sex function, maintenance, and growth compete for the energy supplied by the diet. A carefully considered investigation of the part played by growth in influencing the oestrous cycle in vitamin-A deficiency has been reported by Mason & Ellison (98). Using the supravital method of staining the cells in vaginal lavages, it was shown that in the rat, in marked vitamin-A deficiency with persistence of cornified cells, there still is an oestrous cycle. Basing their observations on a series of cross-controlled groups of rats with inanition and vitamin-A deficiency as variants, the authors showed that the length of oestrous cycles, and therefore the number per unit of time, is a function of inanition, whereas the character of the cells obtained in the lavage varies with the deficiency of vitamin A. The irregular and prolonged oestrous periods observed in vitamin-A deficiency can thus be referred to an indirect effect of the deficiency, i.e., to the accompanying inanition.

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DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY
YALE UNIVERSITY
NEW HAVEN, CONNECTICUT

LIVER AND BILE*

BY ANDREW C. IVY AND LATHAN A. CRANDALL, JR.

*Department of Physiology and Pharmacology
Northwestern University Medical School, Chicago*

The task of classifying original communications is obviously more difficult when one deals with an organ of diversified functions rather than with a single phase of metabolism. It has therefore been necessary to include certain contributions under a "General" heading. The literature, as usual, is replete with projected tests for liver function; these have not been discussed except where they involve new findings of importance in our conception of the various activities of the liver. With this exception we have attempted to survey the literature with some thoroughness, omitting for lack of space some articles which are confirmatory or which would be duplicated in other sections of the *Review*.

Correlating recent major contributions without attempting to be comprehensive, Best (1) has discussed the rôle of the liver in the metabolism of carbohydrate and fat. Greene, Bercovitz & Hanssen (2) have surveyed those developments that are primarily of clinical interest, with emphasis on tests applicable to the estimation of liver function. In a monograph on the results obtained in experiments using the "angiostomy" technique, London (3) has included many observations on the various functions of the liver; unfortunately he does not, in most cases, give absolute values for the blood concentrations of various substances but is content to tabulate differences between the blood entering and leaving the organ under study. Forsgren (4) has presented a monograph on the rhythmicity of liver function.

CARBOHYDRATE METABOLISM

Bell & Young (5) report on the identity of the glycogens obtained from rat, rabbit, and fish. Bell & Kosterlitz (6) state that rabbit and fish glycogens do not appear to undergo degradation when acetylated or benzoylated, and that the glycogen regenerated from these derivatives seemed to have undergone no change. The content of organic

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phosphorus was very small in the original material and was further decreased on regeneration of the derivatives. Bell (7) methylated fish and rabbit glycogens and found them to have the same chemical structure. Hydrolysis of the fully methylated glycogens, followed by a quantitative separation of the cleavage products, gave tetramethylglucopyranose, 2,3,6-trimethyl-glucose, and dimethyl glucose, showing that the glycogens from both sources are built up of unbranched chains of 12 glucose units. Willstätter & Rohdewald (8) found that the ratio of the glycogen that could be extracted from the liver with water to the total glycogen varied with the nutritional state of the animal. If the glycogen content is high a high percentage is extractable; if low, most of it is bound to protein. The "desmo" fraction can be liberated with difficulty by the action of protease. Purves (9) states that the glycogen and free sugar of rabbit liver fail by 25 per cent to account for the total fermentable carbohydrate present. Attributing the deficiency to a fermentable fraction which was destroyed during the glycogen determination, he isolated from the liver in a yield of 0.07 per cent a carbohydrate that was free from glucose and glycogen and that was easily fermented by yeast; it appeared to be a mixture of dextrans.

Kerly & Reid (10) have found that in both intact and splenectomized cats the liver forms glycogen as well from glucose given by vein as from that administered orally; this is also true for lactic acid administration. Neither acetylcholine nor choline increases glycogen formation under these conditions. This is contrary to the finding of Grant (11) that splenectomy suppressed the formation of glycogen when ammonium lactate was infused into the superior mesenteric vein, and that simultaneous administration of acetylcholine and ammonium lactate to the splenectomized animal restored glycogenesis. Upon decapitation in cats the liver glycogen first falls and then rises; the same is true of the other carbohydrate constituents of the liver which leads Tsai (12) to believe that glycogen is not regenerated at the expense of other carbohydrate. However, the blood sugar fell before the increase in liver glycogen appeared.

Butsch (13) injected dogs with 10 per cent dextrose solution intravenously at rates between 3 and 5 gm. per kg. per hour. After thirty-six to fifty-one hours there was an abrupt decrease in the amount of dextrose retained; this corresponded with the deposition in the liver of about 20 per cent of its weight as glycogen, being the maximum that it would retain under these conditions. According to Salter,

Robb & Scharles (14) the amount of glycogen deposited in fasting mice is roughly proportional to the amount of carbohydrate fed; glucose, sorbitol, gluconate, glucosamine, ethyl and methyl glucosides, and propylene glycol all increased liver glycogen, methyl glucoside being toxic. Reid (15) found in chloralosed cats fasted twenty-four hours and in which the body temperature was maintained at 38°, that liver glycogen disappears at the rate of 0.1 per cent per hour. At a temperature of 40° or more there is a slight increase in the rate of disappearance, while below 37° glycogen may even be deposited. In the heart-lung-liver preparation Bassani (16) observed that the blood sugar steadily decreased to a value of 20 mg. per 100 cc. He concluded that the deposition of liver glycogen is proportional to the concentration of glucose in the blood under these conditions. According to Abelin (17) fat diets favor the deposition of liver glycogen in rats unless the fat fed is cod-liver oil; frequent interruption of the fat diets by sugar feedings decreases glycogen deposition. Stone (18) studied the effect of high-carbohydrate, high-fat, high-protein, and mixed diets on liver regeneration and glycogen content after removal of 70 per cent of the organ in rats. The most rapid regeneration occurred on the high-carbohydrate and mixed diets, which also produced the largest amounts of glycogen. A further interesting observation made in these experiments was the abrupt fall of liver glycogen when the animals that had been receiving high-carbohydrate diets were starved for brief periods; this produced the lowest glycogen values that he observed. Such marked decreases on starvation were not seen in the rats that had been receiving other diets.

Goldblatt (19) states that either insulin or epinephrine in proper dosage may produce an increase in liver glycogen in the starving young rabbit; the injection of insulin was not accompanied by such an increase in blood lactic acid as would indicate that an increased secretion of epinephrine from the adrenals was responsible for the rise in glycogen content. Kosterlitz (20) reports that glucose administered to pancreatectomized dogs does not cause an increase in liver glycogen, while levulose and *d*-sorbitol produce some deposition of glycogen. It should be noted that Major and later Major & Mann in 1932 found that small amounts of glycogen could be deposited in depancreatized dogs when glucose was given intravenously. Kuramoto (21) and Watanabe (22) both report that cholic acid stimulates the deposition of liver glycogen. Kuramoto finds that a combined injection of epinephrine and cholic acid has the same effect, but that an increase

in the amount of cholic acid given reverses the effect. Watanabe states that the glycogenic effect of cholic acid is antagonized by adenylypyrophosphate.

Gulick, Samuels & Deuel (23) found that removal of the ovaries increases glycogen storage and subsequent injections of theelin reduce the glycogen content, although not to the pre-operative level. Englehart & Riml (24) state that injections of pregnancy urine or corpus-luteum extracts are also very effective in causing glycogen storage. In confirmation of previous investigators, Schneider & Widmann (25) find that thyroxin causes a marked decrease in liver glycogen; thyreotropic hormone acts similarly.

With growing interest in the rôle of the pituitary and adrenals in carbohydrate metabolism has come increasing evidence that the liver must be fundamentally involved in the changes produced by removal of these glands. As yet, much of this evidence is indirect, dealing with the ability of the body as a whole to maintain the blood sugar by the conversion of glucose precursors. Since the liver is the chief if not the sole site of gluconeogenesis such studies are indicative of changes in liver function. At present much of the evidence is contradictory. Only those reports which specifically deal with the liver will be cited. Holden (26) found that the injection of anterior pituitary extracts is followed by a decrease in liver glycogen which returns to a sub-normal level even though the administration of the extract is continued. Holdum & Thurston (27) confirmed the decrease in glycogen, and found that it is not prevented by potassium iodide, which in itself causes some decrease. Fluch, Greiner & Loewi (28) state that removal of the anterior pituitary in frogs does not affect the glycogen concentration, although it decreases the degree of hepatic glycogenolysis that is produced when the livers are perfused with weak epinephrine solutions. According to Campos, Curutchet & Lanari (29) the presence of the liver is essential for the hyperglycemic action of anterior pituitary extracts.

Relations between glycogen storage and other liver functions have been observed by a number of investigators. Ikushima (30) states that the excretion of dyes by the liver is somewhat depressed when its glycogen content is low. This finding is supported by the observation of Minibeck (31) that liver function as tested by the excretion of hippuric acid or santonin, or the elimination of rose Bengal, is depressed by a high-fat diet and improved by a diet high in carbohydrate. Scharles, Robb & Salter (32) report that in general any

change in glycogen concentration is associated with an increased amylolytic activity of liver extract. They tentatively conclude that an augmented enzyme activity regularly accompanies the storage or release of liver glycogen. Walthard (33) found that the oxygen consumption of rat liver in the Warburg apparatus is decreased by the presence of a high glycogen content when this is native to the liver; artificially-added glycogen has no effect. MacKay & Bergman (34) find that 3.6 to 4.4 gm. of water are stored with each gram of glycogen and 2 gm. of water per gram of protein, but that none is stored with fat. Greisheimer & Goldsworthy (35) state that there is a high degree of correlation in rats between the absolute amounts of glycogen and water in the liver, but not between the percentage of glycogen and the percentage of water. There was no correlation between the blood-sugar level and the liver water or glycogen. Delhougne (36) observed that a decrease in liver glycogen produced by phosphorus, toluylenediamine, bile-duct ligation, or phlorhizin entailed a lowered concentration of glutathione in the organ; this was presumed to be indicative of a decrease in catalytic processes. London (3) discusses at some length the occurrence of glycogen in the blood, and concludes from evidence accumulated in his laboratory that the liver continually liberates glycogen into the hepatic venous blood; it is then removed by the skeletal muscles and the intestines.

Tsai & Yi (37) studied portal and hepatic blood-sugar concentrations after decapitation in cats, and found that hepatic venous sugar was always higher than portal, even when the liver glycogen was increasing and even during the absorption of glucose from the gut. Similar observations were made by Olmsted & Read (38). Giragosintz & Olmsted (39) continued these investigations in dogs under amytal anesthesia, with and without ligation of the hepatic artery, after the subcutaneous injection of glucose or during absorption of this sugar from the intestine. They again found that the hepatic venous sugar is always higher than that in the portal vein, even while glycogen is apparently being formed. The difference between the lactic acid content of the hepatic and portal bloods was not sufficient to account for more than half the glucose added to the blood by the liver. All of these investigators concluded that it is unlikely that liver glycogen can come directly from the dextrose of the blood, but must be formed by some other glycogenic substances. It should be noted that while London (3) does not discuss this point in his recent monograph, he includes data (page 73) which indicate retention of

dextrose by the liver of the unanesthetized animal during the absorption of glucose from the intestine. It seems quite possible that the decapitation or amytal anesthesia may be responsible for the observed differences.

Evans (40) exposed rats to decreased pressures ($\frac{1}{2}$ atm.) and found that the glycogen of the liver increased without a concomitant decrease of carbohydrate elsewhere in the body. This effect was dependent upon the integrity of the adrenals, and was interpreted as gluconeogenesis from protein or fat. When the liver glycogen of the rat was reduced by fasting and the blood pressure then decreased below 45 mm. of mercury, Kosterlitz (41) found a decrease in the blood sugar which could be restored to normal by elevating the blood pressure with intravenous injections of acacia. In well-fed rats a similar hypotension produced hyperglycemia; Kosterlitz attributed the hypoglycemia of the fasted animals to decreased gluconeogenesis. Diurnal bimodal changes of glycogen and water content in the livers of pregnant rats essentially similar to those occurring in normal animals are described by Goodwin & Higgins (42); the peaks occur somewhat earlier in the pregnant animals than in normals; the amounts of glycogen were consistently less in pregnant animals, and the peak amounts of water were always greater early in pregnancy than at term.

Sterkin & Vengerova (43) demonstrated that levulose given orally or intravenously causes a marked rise in the blood lactic acid, while glucose, lactose, and maltose have no effect. According to Wierzuchowski & Sekuracki (44) the liver is the principal organ liberating lactic acid during fructose metabolism, since the hepatic vein contains 6 to 12 mg. more lactic acid per 100 cc. than does the blood entering the liver. The arterial lactic acid may rise as high as 36 to 67 mg. per 100 cc. after levulose. Production of lactic acid by the liver, as determined by analyses of portal, arterial, and venous blood, has also been described by Kato (45) in experimental hyperthyroidism, and by Izumi (46) in experimental ileus. Kato states that the intravenous injection of lactate increases arterial, portal, and venous lactic acid concentrations without affecting their relative magnitudes. Bott & Wilson (47) determined lactic acid in blood and liver samples taken at the same time, and found that the concentration is always less in the liver than in the blood; they apparently did not calculate their data in terms of 100 cc. of water in liver and blood and it may be questioned whether the concentrations in terms of water content would not be nearly equal.

Soskin, Allweiss & Cohn (48) and later Soskin & Allweiss (49) have presented evidence to show that the form of the blood-sugar curve obtained after the administration of dextrose is dependent upon the liver and independent of the pancreas. Depancreatized dogs receiving just sufficient insulin and dextrose at a constant rate by intravenous injection to maintain a normal blood-sugar level exhibit entirely normal sugar-tolerance curves. In hepatectomized dogs receiving dextrose intravenously by constant injection so that the blood-sugar level was maintained, the administration of additional dextrose resulted in a "diabetic" type of curve. This eliminates the necessity for postulating abrupt changes of insulin output in the regulation of the blood-sugar level, and is compatible with recent clinical results, which indicate that the diabetic patient is as well or better controlled by the injection of small doses of insulin every two hours as by larger amounts given just before the ingestion of food. Soskin & Mirsky (50) studied the effect of progressive liver injury upon the form of the dextrose-tolerance curve and describe three stages: (a) a "diabetic" curve, attributed to a decreased inhibitory effect of the dextrose upon gluconeogenesis, (b) a normal curve when this factor ceases to operate, and (c) a "supernormal" curve due to increased inhibition of gluconeogenesis. A terminal stage in which large amounts of dextrose may yield "diabetic" curves and small amounts "supernormal" curves is also described. Similar "supernormal" curves indicating increased tolerance have been found by Althausen, Blomquist & Whedon (51) after reticulo-endothelial blockade. Since these investigators do not consider that the liver parenchyma was damaged as a result of the blockade, they postulate that the reticulo-endothelial cells function in the transmission of glycogen precursors from the blood to the parenchymal cells and that the blockade increases tolerance by decreasing the material available for gluconeogenesis. It is perhaps more probable that reticulo-endothelial blockade may affect the functions of the parenchymal cells themselves. The histologic normality present even after blockade cannot be regarded as an adequate criterion of the functional adequacy of the liver parenchyma. In his conclusion that the glucose-tolerance curve is not an indication of hepatic function, Schmiedt (52) confirms clinically these observations on the rôle of the liver in the disposal of exogenous glucose. He finds that the behavior of the respiratory quotient after levulose administration is characteristic of hepatic disease. Further Sprague (53) reports that Eck-fistula dogs are slightly but significantly more

sensitive to insulin than are normal animals, which coincides with the conception that in chronic liver injury, with decreased gluconeogenesis, the carbohydrate tolerance should be increased. Such an increase in dextrose tolerance in Eck-fistula animals has been the subject of a preliminary report from this laboratory (54).

Drury & Salter (55) have studied the efficacy of a number of glucose derivatives in prolonging the survival time of hepatectomized rabbits. The substances were: sorbitol, ethyl glucoside, gluconic acid, glycuronic acid, gulonic acid, saccharic acid, glucosamine, levulose, glucosone, 3-methylglucose, thioglucose, glucose monocarbonate, glycerol, glyceraldehyde, pyruvic acid, propylene glycol, dihydroxyacetone, ethyl alcohol, and ethylene glycol. Most of these can be utilized by the intact animal, but except for levulose and a few anomalous results with propylene glycol and dihydroxyacetone prolongation of life was not observed, nor was the blood-sugar level increased above that of the controls. The utilization of galactose in hepatectomized dogs has been studied by Bollman, Mann & Power (56), who found that while the blood-galactose curves for normal and hepatectomized animals are very similar (the hepatectomized being slightly higher), 10 to 30 per cent was eliminated in the urine of the normal dog as compared with 50 to 60 per cent after hepatectomy. They believe that definite amounts are utilized in the absence of the liver, although it does not appear to be converted into glucose for the blood sugar is not increased. The removal of 50 to 70 per cent of the liver had no effect upon the excretion of galactose, but the loss of this sugar in the urine was greater after poisoning with carbon tetrachloride, phosphorus, chloroform, or toluylenediamine.

From experiments upon rat-liver slices Cedrangolo (57) concluded that pyruvic acid was completely oxidized. Cori & Shine (58) found that such slices incubated with α - or β -glycerophosphate or with glycerol produced more fermentable carbohydrate than the controls, the glycerophosphates being more effective than glycerol. Noltie (59) found that the rate of glycogenolysis in excised rabbit liver does not depend upon the amount of glycogen present, nor do the lactic acid and free sugar appearing account for the amount of glycogen which disappears. In a later article (60) Noltie states that iodoacetate does not prevent glycolysis in liver slices, but slightly inhibits the accumulation of sugar and prevents any change in the concentration of lactic acid. From "angiostomy" experiments London, Ivanenko & Prokhorova (61) conclude that pyruvic acid may

be formed or removed by brain, intestine, muscle, and kidney; they believe that methylglyoxal is also an intermediary in carbohydrate metabolism being formed or removed by the above organs and by the liver.

Of considerable interest are reports that citric and oxalic acid excretion are increased when the liver is injured. This perhaps should be considered as evidence of the detoxifying function of the liver. Both, however, have been known to be normal to the body fluid, citric acid having been found in considerable quantities in milk and oxalic being present in blood and urine. Boothby & Adams (62) have found that the output of citric acid in the urine, normally about 0.03 gm. daily, may increase to as much as 2 gm. after hepatectomy. In this connection it is interesting that Langecker (63) observed that liver and kidney decompose citric acid enzymatically, while other tissues have little or no effect. De Lucia & Fucci (64) and Fortunato (65) have reported upon the increased excretion of oxalic acid in the urine of patients with hepatic disease.

LIPID METABOLISM

In a series of articles Best and his coworkers (66, 67, 68, 69, 70, 71, 72) have discussed the action of choline¹ on liver fat. It would appear (67) that the fatty liver which results from a diet high in fat is the result of an excessive deposition of triglycerides, but on a high-cholesterol diet the liver contains increased amounts of both neutral fat and cholesterol esters. The phosphatide fraction varies inversely as the total lipid. Choline was found to be effective in the prevention of both types, and its addition to the diet increased the phosphatide percentage but did not increase the proportion of phosphatides that contain choline in the molecule, nor did it have any effect on the degree of unsaturation of the fatty acids. A low-choline diet containing 40 per cent of fat resulted (69) in a decreased rate of weight gain in rats as compared with controls on the same diet with added choline. The choline group showed a lower mortality and their fur was in better condition. It was noted (70) that diabetic dogs not receiving added choline may show a decrease in glycosuria at a time when the liver has not yet become fatty. It is presumed that both the fatty liver and the decrease in the sugar excretion are consequences of choline

¹ Cf. also this volume, p. 241. (EDITOR.)

lack, and are not necessarily related to each other. An increase in the rate of disappearance of liver fat after phosphorus poisoning was observed (71) on feeding choline, but choline had no inhibitory effect on the fat deposited as a result of phosphorus poisoning. Diets which were deficient in choline led to an accumulation of liver fat which was less rapid than that which followed the administration of phosphorus; if phosphorus-poisoned animals were kept on a low-choline diet the accumulated liver fat did not decrease when the phosphorus was withdrawn. Rats with fatty livers produced by cholesterol feeding showed a rapid fall in triglycerides and a delayed fall in cholesterol esters when given choline (72). As a general conclusion, this group of investigators believe that choline may prove to be a dietary essential. The mechanism by which it prevents the accumulation of liver fat or hastens the disappearance of such fat is still obscure.

The liver fats in depancreatized dogs receiving a diet of meat, sucrose, bone ash, and vitamin sources were studied by Kaplan & Chaikoff (73). No source of choline was added to the diet. Although in the post-absorptive state the blood fats of these dogs were always below normal, enormous amounts of fat were present in the liver. This appeared to account largely for the increase in liver weight, which was 7.2 to 10.8 per cent of the body weight as compared with 1.9 to 2.8 per cent in normal animals. About 97 per cent of the accumulated lipid was in the form of triglycerides; phospholipid accumulated slightly, if at all. Cholesterol almost doubled and the ester fraction was 45 to 88 per cent as compared with 17 to 28 per cent in normal animals; at the same time the percentage of cholesterol esters in the blood decreased. In a later report Chaikoff (74) states that depancreatized dogs can survive for four and a half years on the above diet. In addition to the increased liver fat and the decrease in blood lipids, such animals exhibited opacities of the lens; the general appearance of the animals was that of normal dogs. The essentiality of choline in the diet is questioned.

Channon & Wilkinson (75) state that except for the triglyceride fraction, choline has no obvious effect on the liver lipids. The effect on rats of a diet containing 40 parts of sucrose, 20 of caseinogen, 40 of fat, and 2 of cholesterol, with and without the addition of choline, was studied by Aylward, Channon & Wilkinson (76). With added choline the phosphatides reached a minimum seven hours after feeding and rose to normal after thirteen hours. The neutral fat began to rise in four hours, reached a maximum at ten hours, and was normal

in thirteen hours; the iodine number fell until the tenth hour; the cholesterol remained unchanged but the cholesterol esters increased linearly after the seventh hour. It was shown that choline lessens the decrease in phosphatides and decreases the accumulation of neutral fat. In a second communication, Channon & Wilkinson (77) describe the effect of varying the proportions of protein and carbohydrate in a diet containing 40 per cent of fat and no added choline. Naturally occurring choline in the diet amounted to 1.5 to 2.0 mg. per day. The amount of fat deposited in the liver was found to be conditioned by the proportion of protein, being low when much protein was fed and higher when the amount of protein was low. Best, Huntsman & Ridout (78) later point out that the amount of protein naturally occurring in the diet used by Channon & Wilkinson is sufficient to exert a distinct "lipotropic" effect; while they do not deny the possibility that protein may be "lipotropic," they believe that this question can be finally determined only by feeding highly purified proteins or amino acids. The possibility that certain protein fractions may be converted to choline is discussed. Mukerji & Van Dyke (79) reported that choline fed to rabbits for three weeks or more in doses of from 200 to 500 mg. per kilo daily did not prevent the acute increase in liver fat that follows a single large dose of pitressin. Blatherwick *et al.* (80) describe fatty infiltration in the liver when a diet of whole liver is fed, and consider that the cholesterol content of the ingested liver may be responsible. They did not find a deposition of fat when liver extract was administered. Lecithin did not inhibit the deposition of fat on whole liver diets.

Selye, Collip & Thomson (81) have produced a deposition of fat in the hepatic tissue remaining after removal of 70 per cent of the liver by procedures that disturb the fluid balance of the body. Such partially hepatectomized rats do not develop an increase in liver fat spontaneously, but it does occur when the drinking water is withheld, after hemorrhage, or following the intravenous injection of saline in amounts equivalent to 10 per cent of the body weight. The deposition of liver fat in these animals can be prevented by adrenalectomy, or by an hypophysectomy performed some time previously. Dible & Libman (82) have shown that the degree of fatty infiltration occurring in the liver during fasting depends upon the amount of fat present in the body. Sperry & Stoyanoff (83) state that chickens, like mammals, deposit cholesterol in the liver on a high-cholesterol diet; but unlike rats they deposit more free cholesterol in proportion to the total. A diurnal variation in the neutral-fat content of the

liver has been described by Ohlsson & Blix (84); it is independent of food, the fat content being high in the early morning, decreasing in the afternoon, and appearing to be somewhat reciprocal to the glycogen content. They found no cycle in the phosphatide or water percentage. According to Sinclair (85), elaidic acid may comprise as much as one-third of the total fatty acids in the liver phospholipins of rats on diets high in elaidin; this is also true of muscle. The rate of change when the animals are placed upon the diet is rapid in the liver (one day), and requires many days for muscle.

Lajos (86) found that the administration of insulin causes a decrease in the neutral fat and an increase in phospholipids of both liver and blood; the action of adrenalin was directly opposite. According to Barrett & Wilson (87), adrenalectomy is without effect upon the nature or amount of the liver fat. The injection of thyroxin in rabbits was found by Schmitt (88) to be followed by a decrease in liver phospholipid and an increase in that of the muscle. Non-phospholipid fatty acids increased in the liver and decreased in muscle. They believe that these changes are compatible with the theory that the phospholipids participate in the intermediary metabolism of fat. Franke & Malezynski (89) observed that β -hydroxybutyric acid increased in hepatectomized animals as long as they remained alive, although little or no acetone or acetoacetic acid appeared.

Mazza (90) reports that liver extracts oxidize palmitic and stearic acids, and act slightly on lauric, but have no effect on formic, acetic, butyric, or caproic. Thus extracts differ from liver slices which oxidize all but formic acid. Quastel & Wheatley (91) studied the oxidation of fatty acids by liver in the Warburg apparatus; all except formic acid markedly increased respiration. The acids with an even number of carbon atoms all gave rise to acetone bodies in considerable amounts, while the acids containing odd numbers of carbon atoms produced little or no ketone. Acetoacetic acid was not appreciably broken down to acetone, and is apparently the end product of butyric acid oxidation. Jowett & Quastel (92) found the rate of acetoacetic acid production to vary as a function of substrate concentration with butyric and β -hydroxybutyric acids under similar conditions. The optimum pH was about 7.4; changes in potassium- and calcium-ion concentrations altered the rate of acetoacetic acid production. Benzoates, cinnamates, and phenylpropionates at low concentrations appeared to inhibit fatty acid oxidation specifically. Acetoacetic was

probably the only β -ketonic acid produced in significant amounts. Acids of 4, 6, and 8 carbon atoms produced acetoacetic most rapidly. Acids of 5, 7, and 9 carbon atoms produced small but significant amounts of acetoacetic acid and the increased respiration of the liver slices suggested that they are oxidized more completely than the acids with an even number of carbon atoms. These and other observations, they believe, are incompatible with the theory of beta-oxidation, for which they substitute a conception of "multiple alternate oxidation." Edson (93) has confirmed the report of Annau (94) that the presence of ammonium chloride increases the production of acetoacetic acid from pyruvic acid in the presence of liver slices. In the absence of any substrate Edson finds that ammonium chloride will increase the formation of acetoacetic acid if the liver sections are taken from a well-nourished animal but has no effect on those from a fasting animal in which the rate is already high. Ammonium chloride thus reduces the difference between acetoacetic acid production in livers from fasting and fed animals. He also finds that fatty acids of both even and odd numbered carbon atoms yield β -ketonic acids, the yield from the even compounds being about three times that of the odd. The effect of ammonia was inhibited by the presence of glycerol. The action of choline on the metabolism of liver slices was studied by Trowell (95), who states that it is apparently oxidized by the addition of one atom of oxygen to the choline molecule. Choline inhibits acetoacetic acid production and therefore, presumably, the oxidation of fat. The increase in oxygen consumption when choline is added is, therefore, the algebraic sum of the uptake of oxygen due to its oxidation and the decrease due to suppression of fat oxidation. Gemmill & Holmes (97) found that the respiratory quotient of liver slices from rats fed a normal diet was 0.75 and from those on a diet of butter 0.58; in the latter case the carbohydrate content of the slices increased on shaking for three hours in Ringer's solution at 37°. They consider that a conversion of fat to carbohydrate is indicated. Cori & Shine (58) take exception to this conclusion. Irving & Smith (96) present a rather complete analysis of the fatty acids of pig liver; 35.6 per cent were saturated.

METABOLISM OF NITROGENOUS COMPOUNDS

There are relatively few original contributions concerning the rôle of the liver in the metabolism of amino acids or other protein

derivatives, although numerous reports dealing with the testing of known functions as an aid in the diagnosis of hepatic disease have appeared. Nakao (98), Monguio & Krause (99), Wagner & Gneiting (100), and Sievert (101) among others have discussed hepatic function tests based on tolerance to amino acids. Nakao further states that while the fasting level of the blood uric acid is not changed in patients with hepatic disease, such individuals show a delay in the changes in blood uric acid that normally follow the ingestion of peptone. Much interest is evinced in the Takata-Ara test for hepatic function. The most extensive publication on this subject is that of Hafström (102). The test appears to depend upon changes in the proportions of the blood proteins, specifically the albumin-globulin ratio, and may be positive not only in hepatic disease but also in nephritis and various types of respiratory infections; it may be tentatively accepted as further evidence that the liver is concerned in the maintenance of normal blood-protein relationships.

Neber (103) has described the formation of amino acids by liver slices acting on a substrate of keto acids and ammonia. This is not a new conception, having been previously demonstrated by feeding experiments (Knoop). The synthesis is possible only in the presence of intact liver cells, and proceeds most rapidly when liver glycogen is high. Ornithine appears to act as a regulator, slowing an initially high rate and increasing the rate when it was previously low. London *et al.* (104), on the basis of the hepatic vein output of urea after the injection into the portal of ammonium chloride in combination with citrulline, ornithine, or arginine, disagree with the theory of Krebs concerning the mechanism of urea formation. They found that only ornithine increases urea formation. Russo (105) perfused avian (fowl) liver with ammonium salts. Urea was not formed in these experiments, but a production of uric acid was noted. He therefore believes that urea does not constitute a stage in the formation of uric acid. Houssay, Deulofeu & Mazzocco (106) studied the conversion of various substances into indoxyl in the absence of the liver or gastro-intestinal tract. Indole and *o*-nitrophenyl acetaldehyde gave rise to indoxyl only in the presence of the liver. Binet & Weller (107) found in guinea pigs that fasting leads to a decrease in the amount of reduced and total glutathione in the liver; the change being first observable on the third day and being pronounced by the eighth.

GENERAL

A considerable number of communications deals with the presence and concentrations of various enzymes in the liver. In most cases these observations are not correlated with the performance of any specific function by the organ, but are primarily concerned with the identification and characterization of the enzyme; such reports will not be discussed.

Several articles contribute to our information concerning the part played by the liver in the regulation of fluid balance and the distribution of the inorganic salts. Torok & Kallo (108) injected hypertonic saline into the portal vein and determined the chlorides in portal and hepatic blood. They conclude that the liver seems to delay the passage of sodium chloride into the general circulation. Vegh (109) states that in liver disease the removal of chloride from the blood following the intravenous injection of sodium chloride is delayed. In a report dealing with the redistribution of fluid after transfusion or intravenous saline, Adolph, Gerbasi & Lepore (110) noted that tying off the liver and spleen decreases the rate at which water leaves the circulation after the infusion of isotonic saline, confirming Lamson *et al.* (1923). Sherman & Barbour (111) gave amidopyrine to dogs previously made febrile by the injection of Shiga vaccine, and showed that during antipyresis the liver gives up much of the water which it stored in the febrile period; amidopyrine had no effect in normal dogs. Adlersberg (112) has presented a clinical and theoretical discussion of the rôle of the liver in water balance.

Krichevskii *et al.* (113) found that colloidal copper causes degeneration and necrosis of the parenchyma of the liver and spleen but has no effect on the reticulo-endothelial system. Andersch & Gibson (114) observed that the intravenous administration of acacia to rabbits and dogs leads to a marked deposition of this material in the liver; little is excreted in the urine. It seems to be very slowly eliminated from the liver and damage to the organ may result if the accumulation becomes excessive. According to MacBryde & Taussig (115), dinitrophenol causes functional changes in the liver (and other organs) indicative of toxicity; this was noted in a large percentage of patients who did not exhibit an idiosyncrasy toward the drug. Calcium lactate was found by Asoda (116) to be protective against liver injury by carbon tetrachloride in rabbits, as determined by changes in the elimination of bilirubin and urobilin from the blood. Loewy (117) states that when pancreatic juice is drained

through an external fistula fatty degeneration of the liver occurs, but when the duct is anastomosed to a ureter the liver appears normal even though the animal loses weight. In view of the accumulation of fat known to occur in the liver following complete removal of the pancreas, this observation should be verified. The pathological changes occurring in the liver in dogs subjected to anaphylactic shock are described by Weatherford (118), and after mechanical obstruction of the hepatic veins by Simonds & Jergesen (119). Stewart, Cantarow & Morgan (120) have reported on renal pathology occurring after biliary stasis and decompression. Boyce & McFetridge (121) discuss liver death and the hepato-renal syndrome. It may be noted that functional and anatomical changes in the kidney are well known in experimental obstructive jaundice and in animals with Eck fistulae.

Drury (122) observed that completely eviscerated rabbits use between 45 and 236 mg. of dextrose per kilo per hour. Pre-operative fasting seems to decrease the dextrose need but has no other effect. The animals die in twenty-seven to forty-six hours without exhibiting the "second stage" symptoms (irascibility, weakness, ataxia, apparent blindness, and increased dextrose need) characteristic of the animal after hepatectomy alone. Drury considers this to be evidence for a detoxifying function of the liver. McNider (123) found that in liver degeneration there is a definitely correlated decrease in the alkali reserve. Field, Leigh, Heim & Drinker (124) state that the specific osmotic pressure of the lymph originating in the liver is lower than that of lymph from other sources, although the albumin-globulin ratio of such lymph is closer to that of serum than is true of lymph from other regions. Matsuda (125) found that there may be a compensatory excretion by the liver of dyes normally eliminated by way of the kidney in the presence of renal injury. Zechmeister & Tuzson (126) present analyses of six human livers for lipochromes: carotene amounts to 0.2 to 2.06 mg. per kg.; lycopin, 0.24 to 0.54; xanthophyll, 0.14 to 0.47; and unknown lipochrome (calculated as lutein) 0.01 to 0.41.

Although frequently omitted from discussions of liver function, the ability of the organ to store certain vitamins is undoubtedly of much significance for the body economy. According to Davies & Moore (127), the amount of vitamin A that can be stored in the liver of rats given a diet rich in this vitamin would be enough to satisfy the minimal requirements of the animal for 1,000 years

(18,000 B.U. per gm.). But on elimination of vitamin A from the diet the amount in the liver fell to 400 B.U. per gram in twelve weeks; there was then no further decrease on continuation of the vitamin-poor diet. Crimm & Short (128) analyzed the livers of seven healthy persons who died from accidental causes, and found an average of 331 I.U. of vitamin A per gram. Two children averaged 80 I.U., while four patients with pulmonary tuberculosis who had been receiving a high-vitamin diet averaged 523 I.U. According to Kuhn, Kaltschmitt & Wagner-Jauregg (129) the liver does not store vitamin B₂ to any extent. Toverud (130) found the ascorbic acid content of the livers from fifty-six newborn Norwegian children to average 7.0 mg. per 100 gm. for the full-term and 6.05 for pre-matures. He discusses the significance of this low content for the early development of scurvy.

BILE

Hawkins & Whipple (131) have recently reviewed the results of many years of investigation on dogs with biliary fistulae. They find that these animals can be maintained in good condition indefinitely on salmon bread plus 50 cc. of dog's bile by mouth daily, provided that the fistula is of such a type that infection of the biliary tract does not occur; the longest survival periods were in dogs with anastomosis of the biliary tract to one ureter. Liver feeding was found to improve the general condition of these animals. If bile is not given by mouth the dogs are subject to: (a) purpura if they live sufficiently long and have complete exclusion of bile from the intestine; it seems to be due to a lack of prothrombin with resultant increase in clotting time; (b) osteoporosis, sometimes complicated by calcium deposition in the lungs and viscera; they consider this as perhaps due to failure of vitamin-D absorption; it may be readily prevented by feeding liver; (c) cholelithiasis; (d) duodenal ulcers; and (e) intestinal intoxication. Cavazza (132) reports that dogs with biliary fistulae show a decrease in blood calcium, an increase in potassium, and a decrease in the alkaline reserve. See also Gray & Ivy (133) for a discussion of calcium in jaundice with a review of the literature. Quick *et al.* (134) found the prothrombin content of the blood decreased in jaundice.

McClure, Huntsinger & Fernald (135) collected human duodenal bile following the administration of cottonseed oil, peptone, or dex-

trose. All of these tended to cause an increase in total fatty acids which was most marked in the soap fraction. Cottonseed oil produced the greatest stimulation, peptone much less, and dextrose very little. Differences between the fats of the bile and of the blood indicated that the lipids of the bile result from secretory activity of the liver rather than being a filtration product. Reinhold & Wilson (136) studied the composition of bile over four to eight-hour periods in dogs with acute biliary fistulae and those of the McMaster & Elman type. The concentrations of the individual cations in bile were usually higher than in serum. The principal anions were bile acids, chlorides, and bicarbonate. The bile acids were quantitatively the most important constituent, although nearly as much phospholipid was present; the two accounted for nearly all of the total solids. Appreciable amounts of undetermined anion are usually present. The total concentration of anions and cations was greater in bile than in serum, although the osmotic pressures of the two fluids are equal; cholates must therefore exert but little osmotic effect or suppress that of other ions. When a decrease of cholate concentration occurred it was partly balanced by a decrease in sodium and partly by an increase in chloride and bicarbonate. The administration of hydrochloric acid made the bile more alkaline because the cholate concentration fell and bicarbonate increased. Sodium bicarbonate intravenously produced little change. Enormous amounts of inorganic salts had to be injected to affect the biliary constituents. Sodium taurocholate given intravenously increased the sodium and cholate of the bile while the bicarbonate decreased; the total anion-cation concentration rose until it was considerably above that of the serum. The choleretic action of sodium dehydrocholate was principally due to an increased excretion of water. Aronsohn & Andrews (137) found that the total, non-protein, and protein nitrogen of human gallbladder bile had no relationship to cholelithiasis or pathologic changes in the gallbladder, nor did ligation of the common bile duct produce any consistent changes. Riegel & Ravdin (138) removed specimens of gallbladder bile from living pregnant women at term, and demonstrated an increase in cholesterol and a decrease in bile salts, which suggests to them both a change in the character of the liver bile and in the absorptive ability of the gallbladder. They discuss the possible relationship of such changes to calculus formation.

Loeper, Lemaire & Tauzin (139) state that folliculin and corpus-luteum extract decrease bile flow. This is confirmed for folliculin by

Baltaceano, Vasiliu & Paraschiv (140), who add that in female dogs anterior pituitary extract increases bile flow, output of bile salts, and decreases the cholesterol content. Tanaka (141) believes that the spleen exerts a hormonal influence over bile and bile-acid production by the liver; after splenectomy the secretion of bile acids and usually that of bile are increased, but the increase disappears if spleen extract or atropin is given. According to Golber (142) thyrotoxicosis increases the cholesterol content of bile and decreases bile acid; the bearing on gall-stone formation is discussed. Periodic secretion of bile was noted by Josephson & Larsson (143) in a patient with a biliary fistula. Asoda (144) reports that the sodium salts of the bile acids when given orally cause a decrease in blood lipoids and an increase in those of the bile.

Josephson (145) has determined the specific rotation and rotary dispersion for cholic, desoxycholic, glycocholic, and taurocholic acids and their salts. Fernholtz (146) reports the isolation of a second ketocholanic acid, 3-hydroxy-6-ketoallocholanic acid, from bile. He suggests that the *allo*configuration results from the saponification process. Aronsohn & Andrews (147) obtained dog's bile without admixture with air or blood. They found the pH lower and the carbon dioxide content higher than has been previously reported. The low concentration of carbon dioxide in gallbladder bile is a reflection of the decreased pH. The lower pH of gallbladder bile is accounted for by an increase in phosphorus content that more than compensates for the decrease in chloride, while total base remains the same, and also by an increase in protein. Bile-acid concentration may also have some effect. Bronner (148) found that in man the pH of the hepatic bile depends on the diet; when the bile is alkaline its calcium content is high, when it is acid the bile salt and fatty acids are high. The iron content of all biles examined was found by Judd & Dry (149) to range from 0.031 to 1.68 mg. per cent, with copper 0.063 to 1.07. Jones & Laing (150) were unable to affect the calcium content of bile by amounts of viosterol which did not increase blood calcium; when the latter rose the calcium of the bile also increased. The output of calcium in the bile was found to depend almost entirely on the volume output. According to Frey (151) the protein osmotic pressure of liver bile is less than that of gallbladder bile, while that of gallbladder bile is about three times as high as in blood. After administration of a cholagogue the protein osmotic pressure of liver bile became negligible. The water and chloride content of gallbladder bile

are inversely related to protein osmotic pressure. Wright (152) found no cholesterol esters in normal dog bile. King & Armstrong (153), using a phenyl phosphate method for phosphatase, find a considerable amount of this enzyme in bile. In a later communication Armstrong & King (154) state that in experimental toxic jaundice (chloroform, phosphorus, or toluylenediamine) the serum phosphatase rose markedly, but remained normal in hemolytic jaundice produced by phenylhydrazine. Herbert (155), in confirmation of previous reports, finds the serum phosphatase high in patients with obstructive jaundice; he observed little or no increase, as a rule, with toxic or infectious jaundice although occasionally the values were above normal; there was no increase in jaundice of hemolytic origin. Armstrong & Banting (156) found no decrease in serum phosphatase after hepatectomy.

Breusch & Johnston (157) confirm the observation of Ravdin that on re-opening the common bile duct after prolonged closure the bile salts do not reappear in the bile for about eight days, although bilirubin is secreted immediately. Aronsohn & Andrews (158) injected 10 to 20 per cent bile salt solutions into the gallbladders of dogs and observed that purified preparations produced inflammatory reactions in 10 per cent concentration. The reaction closely resembled that in human cholecystitis. Nedzvetskii (159) found that cholesterol may be esterified with palmitic, stearic, or oleic acids in the presence of bile salts by the action of lipase. In the absence of bile salts the reaction is extremely slow.

Greaves & Schmidt (160) gave viosterol to rachitic rats, some of which had complete obstructive jaundice, and determined the storage of vitamin D by feeding the livers of these animals. Rats with jaundice and rickets stored the vitamin when it was given subcutaneously, but not when it was fed. Neither oral nor subcutaneous administration of viosterol produced calcification in jaundiced rachitic rats. Greaves & Schmidt believe that the activity of the osteogenic cells may be impaired by the presence of jaundice. The blood calcium in these animals was low; the phosphatase was low in the liver and kidney and increased in the bone. Viosterol would still increase the blood inorganic phosphate. Taylor, Weld & Sykes (161) confirm the observation that the presence of bile in the intestine is essential for the absorption of viosterol. Greaves & Schmidt (162) also present evidence to show that while vitamin A can be absorbed from the intestine in the absence of bile, the absorption of carotene does not

occur. Obstructive jaundice or phosphorus poisoning decreases the ability of the animal to convert carotene to vitamin A. Riegel, Elsom & Ravdin (163) found that fatty acids alone are but slightly absorbed from an isolated intestinal loop. The addition of sodium taurocholate brings about absorption, as does bile to a somewhat less extent. From the proportions of bile salt and fatty acid present as absorption occurred, they conclude that absorption cannot be explained as the result of a chemical reaction between these two compounds, but must be due to a surface phenomenon.

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DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
NORTHWESTERN UNIVERSITY MEDICAL SCHOOL
CHICAGO, ILLINOIS

COMPARATIVE BIOCHEMISTRY OF THE VERTEBRATES AND INVERTEBRATES*

BY F. KUTSCHER
University of Marburg

AND

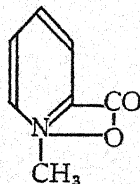
D. ACKERMANN
University of Würzburg

Since the last review on the nitrogen-containing extractives of invertebrates and the lower vertebrates¹ our knowledge has been extended: several such substances which were already known have been observed elsewhere in the animal kingdom; in addition, two new substances have been discovered which had not previously been observed in either the animal or the plant world.

Glycocoll betaine.—This was found, not in large quantities, in *Arca noae* (Ackermann, unpublished work).

Carnitine.—This was found also in *Arca noae* by Kutscher & Ackermann (1), and was identified as the chloroaurate and the ethyl ester platinate. This finding is noteworthy because the compound had previously been demonstrated only once, and that by Morizawa (2) in *Octopus octopodia*. Since γ -butyrobetaine is also found in *Arca noae*, it seems probable that these two substances are genetically related. Carnitine may arise from butyrobetaine by oxidation of the latter in the β -position, or it may be converted into γ -butyrobetaine by partial reduction. Such a process has been demonstrated by Linneweh (3) to be biologically possible as in the case of putrefaction.

Homarine.—Hoppe-Seyler (4) has recently discovered this compound in lobster muscle.



Homarine

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¹ *Ann. Rev. Biochem.*, 2, 355 (1933).

It is the methyl betaine of picolinic acid (α -pyridine carboxylic acid) and is therefore isomeric with trigonelline. Proceeding with the same preparation, Hoppe-Seyler demonstrated that the two substances of empirical formula $C_7H_7NO_2$, found earlier by Holtz, Kutscher, & Thielmann (5) in *Arbatia pustulosa* and by Kutscher & Ackermann (1) in the mussel, *Arca*, were not trigonelline but homarine.

The study of homarine was conducted in the following manner. Several salts of the compound (chloroaurate, chloroplatinate, hydrochloride, and picrate) were first prepared, and these were used in determining the formula of the free base. The constitution was based upon the following evidence: homarine contains one N-methyl group (N-methyl determination) and one esterifiable carboxyl group (preparation of ethyl esters); by heating the dry hydrochloride, vapor having the odor of pyridine was evolved, and by additional heating of the hydrochloride to 200° in the presence of concentrated hydrochloric acid picolinic acid (α -pyridine carboxylic acid) was formed; finally, synthesis of the identical compound was effected by methylation of picolinic acid with dimethyl sulphate.

The occurrence of this betaine in the living organism had formerly been unknown. While the isomer, trigonelline, is formed in many plants, and although the number of complex pyridine derivatives of plant origin is great, there is, in general, only one pyridine base known in the animal kingdom, namely methyl-pyridyl-ammonium hydroxide, which was first found by Kutscher and his coworkers.² It is closely related to homarine and to trigonelline, and could be formed from either by decarboxylation.

With regard to trigonelline, it has been found in the urine after feeding nicotinic acid and also in normal human urine,³ in which case it may have arisen from plant material in the diet. Aside from these occurrences, it has not been found in the animal body; it has, however, appeared much more often in the plant world.

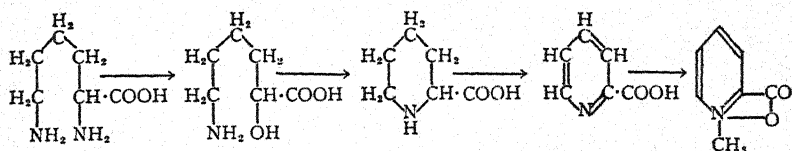
One might think that homarine is formed instead of trigonelline in a number of animal species. If one considers this further, however, it seems probable that both trigonelline and homarine may arise from the same mother substance. As such a precursor, quinoline derivatives would be the most probable. Perhaps by way of quinolinic

² *Ann. Rev. Biochem.*, **2**, 361 (1933).

³ *Ibid.*, **2**, 357 *et seq.* (1933).

acid they might first be degraded to nicotinic acid or to picolinic acid. Quinolinic acid-methyl-betaine would, most probably, be able to give only trigonelline. In plants, a common source of origin would be in the decomposition products of numerous alkaloids; in animals, conceivably, unknown decomposition products of kynurenic acid and of tryptophane.

In the particular case of homarine we must consider the possibility that monohydroxy-monoamino-caproic acid, arising from deamination of lysine, may give rise to α -piperidine-carboxylic acid by ring closure with the removal of water; by subsequent dehydrogenation and methylation homarine could be formed.



Such a series of events would proceed according to the scheme which Drechsel (6) developed to explain the formation of pyridine bases. Klein & Linser (7), for example, describe in a similar manner the formation of stachydrine in plants (from ornithine by way of α -hydroxy-*D*-amino-valeric acid and proline). Since stachydrine is present with homarine in *Arca noae*, it seems probable that in the metabolism of the mussel the two processes (conversion of ornithine to stachydrine and of lysine to homarine) proceed in similar fashion.

Numerous other methods are of course conceivable by which picolinic acid and homarine might ultimately be formed in metabolism from protein-decomposition products. For example, one might derive theoretical relationships between the formation of homarine and the decomposition or alteration of arginine, citrulline, ornithine, proline, and finally glutamic acid, using established methods (α -amino instead of δ -amino-valeric acid), such relations may be traced by suitable adaptations of the scheme for trigonelline formation in plants proposed by Klein & Linser. Heretofore, at least, there is no experimental evidence supporting this method of homarine formation.

With respect to the different general theories pertaining to the formation of pyridine bases in plants (6) it would be important to know whether homarine occurs only in animals and not in plants. Methylated bases, such as betaine, stachydrine, etc., have been dem-

onstrated in lower animals, but in plants above all, they are widely distributed and appear in large amounts. One must always consider the possibility that they have their origin in plant foodstuffs and are retained in the tissue fluids of the animals under observation as seemingly inert substances. Gradually, however, different methylated bases (γ -butyrobetaine, carnitine, trimethylamine oxide, etc.) have been found which, up to the present, are known to occur only in animals, and indeed in comparatively many species, but never, on the contrary, in plants. Quite probably, therefore, they are specific products of animal metabolism. It is likely that homarine belongs in this group. On the other hand, it is easily possible that, hitherto, in working up plant extracts homarine has merely been overlooked or unrecognized. Above all, it may easily be that homarine may have been taken for trigonelline of incomplete purity since the melting points of its salts invariably fall lower than those of the corresponding trigonelline compounds.

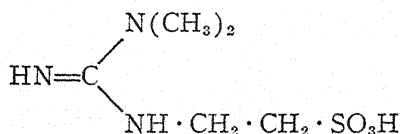
Trimethylamine oxide.—This was recently found by Hoppe-Seyler (8) to be regularly present in the muscle of lobsters (about 0.3 gm. from abdominal muscle). As yet the base has been found only in selachians, teleosts, and cephalopods. It had been supposed earlier by Hoppe-Seyler (9) that the "karnirin," isolated by Suzuki (10) from Japanese giant crabs, and thought by him to be an isomer of lysine, was in reality trimethylamine oxide. This is still plausible because of the discovery of the base in other Crustacea. Small quantities of trimethylamine oxide also seem to occur in the muscle of the fresh water crab *Astacus fluviatilis* (9). This is an important finding, since heretofore the compound has generally been detected only in marine animals, usually teleosts; on the other hand, it has never been found in fresh water teleosts. A demonstration of its occurrence in fresh water crabs could only be accomplished indirectly by determination of the increase in volatile base following upon reduction of extracts with stannous chloride and hydrochloric acid. The material is not present in amounts sufficient for isolation and certain identification.

Trimethylamine.—This base is also present in small amounts in fresh lobster muscle (9). It probably arises from trimethylamine oxide and is responsible for the characteristic taste and odor of the flesh of lobsters and marine fish.

Arginine.—At the same time, Hoppe-Seyler (9) found this amino acid in lobster muscle; the discovery is not surprising. It was isolated through the flavianate, as the copper nitrate salt and the

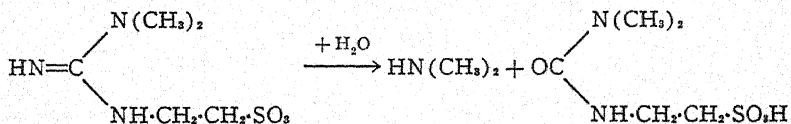
nitrate. It behaved entirely as *d*-arginine, not as the *dl*-form, which Broude (11) obtained from the muscle of fresh-water crabs after complicated fractionation.

Asterubin.—This new guanidine derivative had not been observed heretofore in nature:

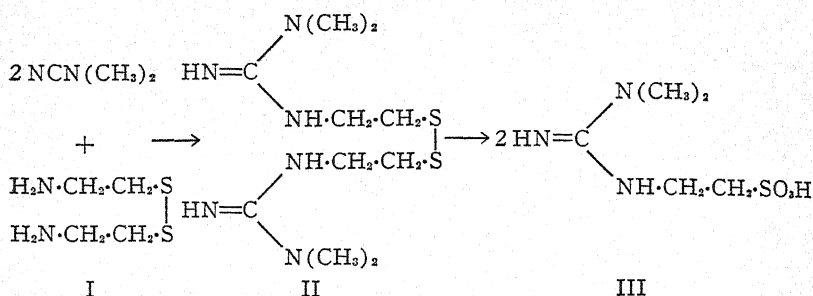


It has now been found by Ackermann (12) in two species of starfish, *Asterias rubens* and *Asterias glacialis*. The substance is readily soluble in water, is optically inactive, and is not precipitated by the usual alkaloidal reagents.

Its constitution, as here presented, is based upon the following relationships: no amino nitrogen is detectable by treatment with nitrous acid; two methyl groups are attached to the nitrogen; sulphur is not detectable by the lead-sulphide test but may be demonstrated by fusion with sodium nitrate; oxidation with permanganate gives guanidine; upon treatment with baryta the molecule is split, with the addition of one molecule of water, into dimethylamine and carbaminylnaurine:



Synthesis may be effected in two different ways (13), either by treatment of taurine with dimethylcyanamide in a sealed tube at 120°, in which case the yield is very small, or by starting with cystamine (I), first prepared by Gabriel. On standing at room temperature this takes up two molecules of dimethylcyanamide. The tetramethyldiguanidyl-cystamine (II) thus obtained is oxidized by hydrogen peroxide in the presence of an iron salt, the reaction proceeding in the same way as that described by Schöberl (14) for the conversion of cystamine into taurine. Thus, the —SS— group goes into two —SO₃H groups and two molecules of asterubin (III) arise:



The structural formula of asterubin leads us now to the following considerations: Whereas it was formerly thought that certain known guanidine derivatives of biological significance, namely guanidine, mono- and dimethyl-guanidine, creatine, creatinine, agmatine, and galegine arose from arginine simply by shortening and modification of the side chain, another interpretation is perhaps called for. This arises from the finding, four years ago, of a diguanidid, so-called arcaine, $\text{HN}:\text{C}(\text{NH}_2) \cdot \text{NH}(\text{CH}_2)_4\text{NH} \cdot (\text{NH}_2)\text{C}:\text{NH}$, which was observed for the first time in the mussel *Arca noae*.⁴ Indeed, Zervas & Bergmann (14a) suggested that diguanidino-valeric acid might be the mother substance of arcaine, since only decarboxylation would be required, and elsewhere under special conditions the authors obtained disproportionate amounts of arginine to ornithine and diguanidino-valeric acid. However, until this hypothetical diguanidino acid has been observed in the living organism, one must seek other possible origins. Among them is the entrance of guanidine residues into molecules of biological significance; this process may be designated as "guanylation." In the case of arcaine, this would be effected by the guanylation of agmatine, which is, in turn, a guanidine derivative of putrescine.

A similar relationship may be observed when one seeks to elucidate the origin of asterubin in metabolism. A sulphur-containing guanidine compound has not been observed heretofore in nature, and therefore we have no alternative hypothesis other than guanylation of a residue, which in this case must be taurine. This is highly probable, as in the case of *Asterias rubens* unchanged taurine could be determined in the presence of asterubin.

There are other examples, also, in which biological guanidine derivatives participate, which are enlightening. After all experi-

⁴ *Ann. Rev. Biochem.*, 2, 368 (1933).

ments have been considered, creatine and creatinine fail to appear as biological decomposition products of arginine; one is obliged to assume the synthesis of its guanidine nucleus. Recently Brand, Harris, and coworkers (15), upon the basis of their experiments with muscle dystrophy, showed that in normal muscle creatine is formed from glycine. In this, either together or in sequence, guanylation and methylation of glycine occur; both processes are similar to those which produce asterubin from taurine.

Further confirmation is seen in the experiments of Krebs & Henseleit (16) with urea formation in the animal body, showing the progression of ornithine through citrulline to arginine. This is an especially important example of guanylation in the vertebrate animal body.

With such frequent observations of methylation at hand, one may attribute a certain latitude to the seemingly rare occurrence of the reaction of guanylation of small molecules; it may be anticipated that future studies in the realm of nitrogen-containing metabolism products will reveal still more examples of guanylation.

In the biological sense guanylation, like methylation, may seem to be a pure supposition. Indeed, merely because guanidine, mono- and di-methyl guanidine, creatine, creatinine, agmatine, galegine, arcaine, and asterubin lack asymmetric carbon atoms they have been considered, to a certain extent, as decomposition products. The accumulation of nitrogen in the molecule by guanylation suggests, moreover, that the deposition of much nitrogen, stored in a small molecule, would be given off to the outside, somewhat as in the case of urea and uric acid.

Pharmacological studies (17) of asterubin showed no effect upon the blood pressure, but an increase in the content of blood sugar.

The possibility of the production of creatine and creatinine from glycine and of asterubin from taurine by guanylation and methylation, directs our attention to several non-basic nitrogen-containing extractives, which as yet have hardly been considered. We must now examine them carefully, for they deserve attention as precursors of the substances described in the previous report.

Glycine.—This is the most important compound of this nature; quantitatively, at least, it is predominant. It was detected early by Chittenden (18) in the mussel, *Pecten irradians*, and later by Kelly (19) in *Pecten opercularis*, as an extractable material.

The identification of glycine in the crustacean *Crango vulgaris* by Berlin (20) is particularly important since here the substance occurs in the presence of large quantities of glycocoll betaine. The theory of Engeland of the origin of betaine by biological methylation of the corresponding amino acid rests essentially upon this co-existence of betaine with its mother substance. Berlin demonstrated the presence of glycine in *Crango vulgaris* by methylation of that portion of extractable material which was not precipitated by phosphotungstic acid, and by isolation of the resulting betaine as the chloroaurate. Even without methylation, however, it is possible to determine glycine in unchanged form, as an unpublished experiment of ours has recently shown. While in *Crango vulgaris* much glycocoll betaine occurs with little glycine, Ackermann (12) was able recently to demonstrate unusually large quantities of glycine in the starfish *Asterias rubens* and with it only small quantities of glycocoll betaine (as yet unpublished). One may therefore surmise that in a few animal species methylation of glycine proceeds to a more or less great extent. In this connection the work of Kossel & Edlbacher (21) is worthy of note. They discovered glycine in the testes of *Strongylocentrotus lividus* and *Astropecten aurantiacus*, and sarcosine (monomethyl glycine) in the radial caeca of *Astropecten aurantiacus*. This last finding suggests that the methylation of glycine proceeds by steps, and that sarcosine represents an intermediate product between glycine and glycocoll betaine.

Among the monoamino acids, in so far as they appear as extractive materials, glycine (and, among the betaines, its resultant glycocoll betaine) appears in quantities so large as to be out of all proportion. The significance of this may be as follows: of all the amino acids, glycine is the only one which has no asymmetric carbon atom; according to the viewpoint first adopted by Pasteur (22), such substances are to be characterized as decomposition materials, as, for example, almost all the constituents of normal urine. The use of glycine for coupling purposes (hippuric acid, glycocholic acid, etc.), as well as the preponderant occurrence of glycine in proteins of low metabolic value, point in the same direction. One may understand from this that upon the decomposition of proteins in the lower animals glycine often remains in large quantities, deposited as betaine, or excreted.

As far as is now known, other amino acids of the protein molecule are less significant, compared with glycine, as extractive materials.

Alanine.—This has been observed in the lobster (23), in the *grossen Krabbe von Echizen* (10), in *Palinurus japonicus* and *Loligo breekeri* (24), in the earthworm (25), and in fresh extract of herring testes (26).

Tyrosine.—Tyrosine has been found in the lobster (23), in the *grossen Krabbe von Echizen* (10), in *Palinurus japonicus* and *Loligo breekeri* (24), *Crango vulgaris* (27), *Asterias glacialis* (12), the flesh of the fish *Auxis tapeinosoma* (24), cyclostomes (28), fresh extract of herring testes (26), and in the *hinteren Speicheldrüse* of *Octopus macropus* (29, 30).⁵

Leucine.—This occurs in the lobster (23), in the *grossen Krabbe von Echizen* (10), in *Crango vulgaris* (27), in *Asterias glacialis* (12), in cyclostomes (28), in fresh herring-testes extract (26), and in *Melolontha vulgaris* (31). In addition, isoleucine has been isolated from the radial caeca of *Astropecten aurantiacus*.

Proline.—This amino acid may be regarded as the mother substance of stachydrine. It has been observed in the lobster (23), and in *Palinurus japonicus* and *Loligo breekeri* (24).

Glutamic acid.—This occurs in the radial caeca of *Astropecten aurantiacus* (21).

Tryptophane.—Tryptophane has been found in the *grossen Krabbe von Echizen* (10), and in fresh extract of herring testes (26).

Phenylalanine.—Phenylalanine is reported to be present in the earthworm (25).

Tyramine (*p*-hydroxyphenylethylamine).—Finally, we must not leave unmentioned the interesting observation of Henze (32) who in 1913 determined this pharmacologically active compound in the salivary glands of cephalopods. He has recently (30) extended this finding by the perfection of a suitable micromethod. The biological conversion of tyramine into tyrosine, which is postulated by Botazzi (29), is not thought to be probable by Henze. Tyramine has been observed only once, in *Lumbricus terrestris* (31).

⁵ The "astacin," found in different organs of fresh-water crabs by Dohrn [*Analecta ad historiam naturalem Astaci fluviatilis* (Dissertation, Berlin, 1861)], would be held by von Furth [*Vergleichende Physiologie der niederen Tiere* (G. Fischer, Jena, 1903)] to be tyrosine of post-mortem origin.

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UNIVERSITY OF MARBURG
GERMANY
UNIVERSITY OF WÜRZBURG
GERMANY

ANIMAL PIGMENTS*

By JEAN ROCHE

*Laboratoire de Chimie biologique, Faculté de Médecine
et de Pharmacie, Marseille, France*

In conformity with the instructions given by the editorial committee, this review is primarily devoted to the respiratory pigments. As in the case of those previously written by Anson and Mirsky,¹ it will be limited to a few questions of special importance in which progress has been made in the course of the past two years. Even with this limitation the whole of the works published on the subjects referred to cannot be considered, but only the most important.

The physiological part of the biochemistry of the pigments participating in cellular oxidations, being discussed elsewhere in the present volume,² has been entirely left aside. The same has been done, for a similar reason, with physiological studies on the bile pigments, on the anemias, the formation of the respiratory pigments and the flavins.

It has seemed to us that the principal progress in the biochemistry of the animal pigments since 1934 resides in the results obtained on their specific or individual characteristics and on their physical state (molecular weight); these two points particularly will be discussed.

HEMATINIC CHROMOPROTEINS (HEMOGLOBINS, ERYTHROCRUORINS, AND CHLOROCRUORINS)

Specificity of the hemoglobins and erythrocruorins.—It is a fact, well established since 1912 by Peters and recently verified by Morrison & Hisey (76) for the pure preparations, and by Fontès & Thivolle (43) for the pigments of anemics, that the combination of oxygen with the hemoglobins takes place according to the ratio, Fe/O_2 , one gram-atom of pigmentary iron per gram-molecule of oxygen fixed. This is a property common to all the hemoglobins. But the hemoglobins of various animals differ somewhat in their affinity for oxygen and in some other characters. From this is derived the notion

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¹ *Ann. Rev. Biochem.*, 1, 535 (1932); 3, 425 (1934).

² Cf. pp. 8, 9.

of diversity of these substances in different species and the concept of specificity of the hemoglobins. During the past two years an endeavor has been made to give a chemical basis to this viewpoint by demonstrating the existence of differences in the composition or the structure of these pigments. It is curious that, simultaneously, the older observations on which this view has been based, the specific immunological properties of these proteins, have been demonstrated as erroneous since, according to Bruynoghe (25), the hemoglobins do not possess any antigenic power. On the other hand, Green, Cohn & Blanchard (50) have contributed some new results on specific characters, showing differences in solubility of the pigments of various animals.

As Poldermann (82) has stated, in the course of systematic studies, all the hemoglobins have the same hematin (protohematin), so it is in the protein constituent that their specificity resides. The globins of different species of vertebrates present slightly different amino acid compositions (93), especially in respect to cystine [Block (21)], and ultraviolet spectra which, in a certain measure, are characteristic of the species [Roche, Dubouloz & Jean (93)]. But it is actually impossible to decide whether the existing differences may not reside also, and even more probably, in the manner of intramolecular combination of the amino acids. Such a diversity of structure would be capable of modifying the number and perhaps the nature of the "hemaffin" groupings [Haurowitz (57)] of globin. This would explain the difference in resistance of the pigments of various animals toward decomposition by bases [Haurowitz (54)]. Unfortunately this is a domain which the present state of protein chemistry does not permit exploring.

All of the globins of the vertebrates have the same type of composition, from which the globins of the invertebrates notably diverge. While the content of the former in arginine, histidine, and lysine is about 4, 8, and 9 per cent, respectively, the latter contain about 10 per cent of arginine, 3 to 4 per cent of histidine, and 1 to 2 per cent of lysine (94). It has been known for a long time (Sorby, Vlès, Anson, Barcroft, Mirsky and Oinuma) that the spectra of the pigments of invertebrates differ notably from those of the vertebrate hemoglobins. On the other hand, Svedberg (105) and Svedberg & Eriksson-Quensel (106) have ascertained that the isoelectric point of the former is in the neighborhood of 5, while that of the latter is always about 7. As with other proteins, the isoelectric point is higher here, inasmuch as the content in diamino acids, principally in

lysine, is greater. For these reasons and for others which will be indicated later, the former are designed by Svedberg as "erythrocruorins" in order to differentiate them clearly from the hemoglobins, which Vlès has already attempted in calling them "protohemoglobins." The name, hemoglobin, ought to be reserved for the chromoprotein of the vertebrates.

Individual characteristics of the hemoglobins of animals of the same species.—On superficial consideration the individual evidences of specificity do not appear very numerous, but it is probable that many manifestations escape us as yet, by reason of their slight intensity.

The differences in crystalline form of the hemoglobins of adult man and of the new-born child were first pointed out by Amantea (17), then observed again by Perrier & Janelli (80), by Nicoletti (78), and by Haurowitz (54). But the relativity in the crystalline form of these pigments prevents attributing to such observations a very great value. Perrier & Janelli have even determined differences in the crystallization of human hemoglobins in relation to race, white or black. Likewise, according to these authors the hemoglobins of various hybrids do not crystallize like those of the animals from which they are descended.

The isoelectric points of human hemoglobins present, according to Glass (47), Glass & Groscurth (48), Tadokoro (108), Geiger (46), and Rubowitz (98) individual variations of some magnitude (for example 6.10 to 6.55 according to Glass), which are related to the sex of the subject (Tadokoro). Nevertheless, it is difficult to accept without reservation the conclusions drawn from these studies.

In the rabbit, Litarcek & Dinischiotu (73) ascertained the existence of important differences in the absorption spectra of the oxy-hemoglobin and the carboxyhemoglobins of normal and anemic animals. A relation was found between these different values and the presence of glutathione, more or less elevated, in the erythrocytes. But this relationship was not maintained when the pure pigment was used in the presence of glutathione (Roche). In another work (72), the same authors have established that the affinity of hemoglobin can present marked differences in various pathological states and at different times in the life of man.

Haurowitz (55) has clearly demonstrated the presence of different hemoglobins in the blood of adult man and in that of the new-born child, thus establishing the existence of individual characteristics

in the hemoglobins. Taking up the older observations of Krüger and of Bischoff, Haurowitz first ascertained that the pure hemoglobin of the new-born child is much more resistant to degradation by bases (transformation into cathemoglobin by splitting-off of the pigment followed by denaturation of the globin) than that of the adult, a finding which could be attributed only to the globin, the hematins being identical. Some analogous observations have been made by Trought (111). Finally, the demonstration that the hemoglobin of the new-born child presents an affinity for oxygen different from that of the adult has been reported by Haurowitz (55) and Hall (53).

The composition of the globins [Schenck (99), Roche, Dubouloz & Jean (93), Lang (68)] and their ultraviolet spectra (93) present among different subjects of the same species notable variations. Moreover, the compositions and the ultraviolet spectra of the globins of the individual pigments can be grouped around a small number of values, each one of these corresponding to several globins; the observed variations are then capable of a certain systematization. In effect, the intensity of absorption at 2,750 Å and the value of the ratio, arginine/sulfur, do not vary in any way whatever between the extreme limits. They present in one series of preparations a number of fairly constant values, corresponding to certain types, and each reflecting without doubt a well-defined molecular organization (9).

Existence of several hemoglobins in the blood of the same individual.—The presence of several hemoglobins in the blood of a single individual is a recent concept.

The kinetic study of the degradation of hemoglobins by bases (von Krüger's reaction), demonstrated from the very first to von Krüger (67), and to Haurowitz (55), that the respiratory pigment of the new-born child is not homogeneous, but is composed of a mixture of a hemoglobin, identical from the point of view of its resistance to bases with that contained in the maternal blood, and of a hemoglobin identical with that of the fetus in its behavior toward the same reagents. Some results of the same kind have been obtained by Brinkmann, Wildschlut & Waterman (22). Incidentally, the hemoglobin of the anemic subject presents the same resistance to the action of bases as that of a healthy man (Haurowitz). As the result of new observations on the affinity of the hemoglobins for oxygen, Haurowitz concludes that there exist in man at least two different hemoglobins: the normal hemoglobin of the adult, the hemoglobin of the fetus, a

hemoglobin which is more resistant to the action of the bases than that of the adult, but different from that of the fetus in its affinity for oxygen. It is possible that there occurs an additional hemoglobin peculiar to pernicious anemia, but this last point is not fully clarified. In the blood of the new-born child there is found 75 per cent of fetal hemoglobin and 15 per cent of adult hemoglobin, the former disappearing progressively in the course of life. The presence of several hemoglobins in the blood of the same animal singularly complicates the study of the specificity of these pigments as individuals. In effect, not only does it require taking into account some differences in the probable composition of these pigments, but also the variable proportion in which they occur in blood in different periods of life and in various pathological states.

We note further that the problem of the individual factors associated with affinity for oxygen is still more complex than that of the existence of a mixture of hemoglobins in the same blood. If, as is probable, two mechanisms are to be taken into consideration for regulating the affinity of hemoglobin for oxygen—the combination of hematin with different globins and modifications in the state of the pigment in the erythrocytes—one can imagine that these two factors will be either additive or compensatory in their effects. This shows incidentally, that one cannot definitely conclude, from changes in oxygen affinity under a given set of conditions, that there is necessarily a modification in composition of the globin.

Muscle hemoglobins.—Some very important progress has been made in our knowledge of the hemoglobins present in the red muscle and the heart of the vertebrates, since Theorell (110) succeeded in obtaining ox hemoglobin in the crystalline state. This hemoglobin has an iron content identical with that of blood hemoglobin (0.34 per cent), which shows that in all of these pigments one finds the same proportion of hematin to globin. Extracted from skeletal muscle, it is found in solution in the state of micelles of which the molecular weight is about 34,000, while the extract of heart muscle contains micelles of two kinds, corresponding to molecular weights of 34,000 and 68,000. The fact that muscle hemoglobin exists in smaller micelles, half as large as those of blood hemoglobin of vertebrates, probably explains its greater diffusibility through the kidney, a fact which has long been well known (Camus). The study of the spectrum of this compound and of its derivatives has been pursued by Theorell, as also its affinity for oxygen and carbon monoxide.

The principal result obtained is the demonstration that its affinity for oxygen is much greater than that of the blood hemoglobins [confirmation of the preliminary observations of Hill (61)], the pH of the medium having, between 8.7 and 6.2, extremely little influence on the dissociation curves of oxyhemoglobin. On the other hand, its affinity for carbon monoxide is much less than that of the blood pigment. In the latter, as in all the blood hemoglobins heretofore studied, the linear relation established by Anson, Barcroft, Mirsky and Oinuma between the relative affinities of the pigment for oxygen and carbon monoxide on one hand, and their spectra (the value of the difference between the α bands of carbon monoxide hemoglobin and oxyhemoglobin or "span") on the other hand, has been verified [confirmation of preliminary observations of Roche (8)].

Because of its affinity for oxygen, it is well to consider this pigment as an important reserve of oxygen at the level of the muscle fibers, a reserve which is very useful for their metabolism in the course of contraction. This point of view is in accord with the fact that the pigment content of various muscles is roughly proportional to their activity, the heart being always the richest among them. Such a function has been definitely attributed to cytochrome. Bechtold (19) thinks that the two pigments are reversibly transformable, one into the other, in muscle, but it seems to us that this opinion should not be accepted. Methods of estimating muscle hemoglobin have been proposed by Shenk, Hall, & King (102) and by Watson (115).

Chlorocruorins.—It has been established by Fox (44) that the combination of oxygen and chlorocruorin in *Spirographis* takes place in the proportion of one gram-molecule of oxygen per gram-atom of iron, as with the hemoglobins. The similarity of the chlorocruorins and erythrocrurins (hemoglobins of invertebrates) first suggested by Svedberg (105), and based by that author on the great micellar sizes of both when dispersed in plasma, has since been supported by Roche & Jean (94) in arguments along chemical lines. In fact, the isoelectric point of *Spirographis* chlorocruorin is 4.5 and the amino acid composition of the protein part of the same substance is very similar to that of the erythrocrurins (94).

Miscellaneous observations on the hemoglobins.—The problem of the combination of hematin and protein in the hemoglobins remains open, in spite of various contributions to the question. Hauro-

witz (57) assumes that the union of the two molecules takes place with the fixation of one carboxyl group of the globin to the iron of the hematin, which would explain why the methemoglobins have an isoelectric point slightly above that of the corresponding globins. It is certain that the hematinic iron ought to participate in the combination, since the porphyrins do not react (Haurowitz). But other elements of hematin ought also to be brought into play since, according to Roche & Bénévent (92), certain hematins of cytochrome-*c* type are not capable of combining with the globins to form hemoglobins. Nevertheless these can form hemochromogens, and it is probable that a different active group in the hematins combines with globin according to whether it forms a hemochromogen or a hemoglobin.

Various other observations have been made, applicable to the hematins and the hemoglobins. Herzog (60) has confirmed and extended his researches on hematoprosthetin (see the review of Fischer & Orth).⁸ The absorption spectrum of the hemochromogens has been studied by Clifcorn, Meloche & Elvehjem (27), by Schönberger (100), and by Hoffmann & Schwartzacher (62); that of the hemoglobins by Drabkin (32), Ray & Blair (83), Adams, Bradley & MacCallum (15), and Shenk, Hall & King (102). Anson & Mirsky (18), pursuing their researches on the denaturation of the hemoglobins, have found that in the presence of salicylates, in neutral solution, hemoglobin undergoes an entirely reversible denaturation. Roughton (97) has followed, thermochemically, the reaction between hemoglobin and oxygen.

The combinations of the methemoglobins with hydrogen peroxide and other peroxides have been studied by Keilin & Hartree (65) and by Haurowitz (56); one molecule of peroxide combines with one molecule of pigment. As with hydrogen peroxide, so also hydrogen fluoride, hydrogen sulphide, and hydrogen cyanide (Haurowitz) are all united by a co-ordinate valence to the hematinic iron of methemoglobin. Finally, Groscurth & Havemann (52), resuming the study of the fixation of carbonic acid on the hemoglobins, conclude that this is a function of certain amino groups which form carbamino compounds capable of dissociation into ions:



In the domain of the comparative biochemistry of these sub-

⁸ *Ann. Rev. Biochem.*, 3, 410 (1934).

stances mention should be made of the researches of Green & Root (51), of Christensen & Dill (26), and of Wolvekamp & Lodewijks (119) on the transport of oxygen by the blood of birds, fish, and frogs. The excellent review of Florkin (1) on the respiratory function of the *milieu intérieur* in the animal kingdom gives a complete presentation of the actual state of this question.

NON-HEMATINIC RESPIRATORY PIGMENTS (HEMOCYANINS AND HEMERYTHRINS)

Hemocyanins.—Conant, Dersch & Mydans (28), pursuing the previous researches of Conant & Humphreys (29), have purified the product obtained by alkaline degradation (Philippi) of the hemocyanin of *Limulus*. In this way they may have been able to obtain a polypeptide with the empirical formula $C_{31}H_{50}O_{15}N_7S_2Cu_2$. This product is a complex of a primary polypeptide, $C_{24}H_{35}O_{10}N_5Cu_2$, with a sulfur compound, $C_7H_{15}O_5N_2S_2$, probably peptide in nature. It contains all the copper of the pigment.

The prosthetic group of *Limulus* hemocyanin would seem to be the "complex cupric salt of a polypeptide composed of three molecules, of serine, one of leucine, and one of tyrosine, and some sulfur-containing compound (28)." In spite of all the interest which this work presents, one should not consider the question of the prosthetic grouping of the hemocyanins solved. In fact, the identity of the product studied and of that presenting a specific absorption in the spectrum of the hemocyanins is not demonstrated, whereas it is for hemocuprin (Schmitz, Laporta, Roche & Dubouloz). Incidentally, if the copper of the pigment is fixed to a complex of polypeptide nature, the significance of this with regard to prosthetic grouping cannot be rigorously compared to that of the hematin; the prosthetic group might then be an actual fragment of the protein molecule but not a compound united with it, as in the case of hematin and globin in the hemoglobins.

The composition of the hemocyanins has been studied by Philippi & Hernler (81), Roche (90), and Roche & Jean (94), and their isoelectric point by Svedberg (105) and Pedersen (79). These researches have permitted a separation of the hemocyanins into two groups: the chromoproteins of the Crustacea and of *Limulus* are characterized by a copper content of approximately 0.18 per cent and a fairly high proportion of diamino acids; those of the molluscs contain 0.25 per cent of copper and are poorer in diamino acids.

Like the protohematin pigments (hemoglobins and erythrocruo-

rins), the hemocyanins, then, are grouped under two types. But it is well to remark that the results on these differ from those obtained with the protohematin pigments: the range of variation in the isoelectric point is slightly less in the hemocyanins and, also, the relationship between the amino acid composition and the value of the isoelectric point is less direct. In fact, one finds among the copper-containing pigments of the crustacean type some chromoproteins with isoelectric points ranging from 4.8 to 6.4 (*Maia*, *Limulus*), although their content in diamino acids is closely similar; some hemocyanins of molluscs, on the other hand, possess an isoelectric point very close to 4.8, while their content in amino acids is much less. These facts constitute a new illustration of the concept that the structure of the chromoprotein is more important for its physico-chemical properties than its composition in amino acids. Finally let us observe that the specific differences in composition in the pigments of the two groups of hemocyanins are of the same order as for the hemoglobins.

In respect to reactions with gases, Root (95) has found that the hemocyanins combine with carbon monoxide to form carboxyhemocyanin, in the proportion of one gram-molecule of gas per two gram-atoms of copper (2 Cu/CO). This ratio is comparable to that which holds in the oxygenation of the pigment (2 Cu/O_2). In the case of the hemocyanins as in that of the hemoglobins, the two gases can be substituted for each other, molecule for molecule. Nevertheless the affinity of carbon monoxide for the copper-containing pigment is much less than that of oxygen for the same substance.

In the domain of physical chemistry, a work of Shack (101) establishes that, according to the zoölogical origin of the hemocyanin and at a pH range approaching that which exists in the hemolymph, reduction or oxygenation has a different action on the fixation of acids and bases. Although for *Helix*, *Maia*, *Octopus* (Roche), and *Homarus* (Shack), the oxygenated form, in the physiological pH zone, fixes more base than the reduced form, oxygenation does not affect the buffer power of the respiratory chromoprotein of *Limulus* (Shack). These facts agree poorly with the generalization of the explanation proposed by L. J. Henderson for the Bohr effect in the hemoglobins, because with certain of the hemocyanins studied (*Helix* and *Limulus*) the Bohr effect is reversed. It is probable that there is a factor, hitherto neglected, which ought to be taken into account in these phenomena.

The molecular weights of these pigments, to be discussed pres-

ently, have been determined by Svedberg & Eriksson-Quensel (106), Svedberg & Hedenius (107), Svedberg (105), and Adair, Adair, Roche & Roche (14). An excellent review on the hemocyanins has been published by Redfield (6).

Hemerythrins.—The pigment of two species has been obtained in the crystalline state. Its constitution and properties have been studied by Florkin (40) and by Roche (89). It is apparently similar to the hemocyanins in respect to its globulin character and in the fact that its prosthetic group is not a derivative of porphyrin. The ultraviolet spectrum of the latter, called hemoferin, is similar to that of hemocuprin; they both lack the peroxidase properties common to the various hematins (Roche). Hemerythrin from *Sipunculus* fixes oxygen in the proportion of one gram-molecule of gas per three gram-atoms of iron (3 Fe/O_2). It does not combine with carbon monoxide (Florkin). The transport of oxygen by hemerythrin in the coelomic fluid of *Sipunculus* has been studied by Florkin (41). The hemerythrins of different species present special characteristics. The molecular weight of that of *Sipunculus* is about 66,000 [Roche & Roche (88)].

GENERAL BIOCHEMISTRY OF THE RESPIRATORY PIGMENTS

Molecular weights and particle size.—In this field results of very great importance have been found, above all by Svedberg and his collaborators, especially Eriksson-Quensel and Hedenius. It is now known that all of the blood hemoglobins of vertebrates have a molecular weight of 68,000. Four methods have furnished concordant data: measurements of osmotic pressure (Adair); sedimentation (Svedberg); diffusion (Anson & Northrop); surface tension (Laporta). The other respiratory pigments have been studied until now only by ultracentrifugation (Svedberg) or by osmotic pressure (Adair and Roche). As a comparison of the data thus obtained will be made later, we shall not present it in detail here.

Without doubt the most remarkable fact among those observed in the course of these works is, on the one hand, the presence within these molecules of a more or less large number of monomers containing one gram-atom of metal, and, on the other hand, the existence of certain of these chromoproteins as particles of enormous size never before encountered in such researches. In addition, an important study relating the distribution of these respiratory pigments and their molecular size has been published by Svedberg (105). It

can be summarized as follows: all the endocellular pigments [hemoglobins and erythrocrucorins (Svedberg), and hemerythrin (Roche & Roche)] exist only as small molecules (of molecular weight 17,000 to 68,000), while the plasma pigments (hemocyanins, erythrocrucorins, and chlorocruorins) are composed of enormous molecules [of molecular weight 300,000 to 5,000,000 (Svedberg; Adair & Roche)]. So far only one exception to this rule has been found: that of the plasma erythrocrucorin of *Chironomus* where the molecular weight is 17,000 or 34,000.

Another very important fact brought out by Svedberg is that the molecular weights of these different pigments vary in geometric progression, following multiples of 34,000, except for certain erythrocrucorins, in which are found particles of molecular weight 17,000. This periodicity, which Svedberg's measurements on a large number of proteins (apart from the respiratory pigments) established as a quasi-generality, has permitted Svedberg to consider that proteins in which the molecular weight is about 34,000 are homomolecular while those of a larger size are aggregates of molecules of size 34,000. In fact these can be dissociated reversibly into smaller molecules. So it is that the erythrocrucorin of *Arenicola*, at a pH above 7.5, dissociates into micelles which attain the size of 640,000, 360,000, 208,000, 138,000, while the normal state of these micelles corresponds to a molecular weight of 2,750,000. Consequently Svedberg proposes the same "unit" for the molecule of size 34,000.

In reality it is probable, as A. Roche has pointed out (86), that this unit is not 34,000, but 17,000, because such smaller molecules (called by Svedberg "half-units") exist in nature (intracellular erythrocrucorins), and among the degradation products of the naturally occurring proteins (Bence-Jones protein) outside of the pH-stability zone. In the case of the respiratory pigments, this fact is of special interest because in the hemoglobins a molecular weight of 17,000 corresponds to one gram-atom of iron (within the limits of experimental error). The physico-chemical unit of the monomer thus coincides with its chemical unit. One must therefore consider the blood hemoglobins of vertebrates, as Adair has long assumed to be true, to be formed by the union of four molecular units.

One might also ask himself whether this unit of Svedberg, the existence of which, as a micelle, seems indisputable, really corresponds to a molecule. It seems that this size has only a physico-chemical meaning and that it should not be attributed to a perfectly

definite chemical individual. This is a fact which, it seems to us, should be studied from the point of view of the plasticity of the protein constituent of the respiratory pigments.

It is remarkable that a small number of erythrocruorins exist in the state of monomers, and yet it is worth while to notice that they are always found with dimeric molecules. In nearly all cases they are in a more complex state than one finds in the chromoproteins. The hemoglobins contain, for a molecular weight of 68,000, four atoms of iron and transport four gram-mols of oxygen. The hemerythrin of *Sipunculus* contains twelve atoms of iron per molecule of 66,000, and transports the same quantity of oxygen as the hemoglobins. This last fact merits emphasis, because it seems to correspond to a general organization of the intracellular transporters of oxygen. With regard to the hemocyanins, they all contain an important number of atoms of metal in their molecules, at least ten atoms of copper transporting five gram-mols of oxygen, in the case of the pigments of certain Crustacea in which the molecular weight reaches 360,000 (the smallest pigments of this type). Even though existence in the form of aggregates seems general among the proteins, this character is particularly marked in certain pigments in which the size of the micelle reaches an order of magnitude never found outside.

Osmotic rôle of the plasma respiratory pigments.—This diversity of molecular size is not without biological interest. The hemoglobins of the higher animals are contained in erythrocytes. Consequently, they are localized in cells which, although in suspension in the plasma, preserve an autonomy comparable in a certain measure to that of the tissue cells. The blood plasma is only the *milieu intérieur*, participating in the exchanges. In the animals with plasma respiratory pigments, on the contrary, the chromoprotein is itself a constituent of the *milieu intérieur*; it is concerned directly with the equilibria between the plasma and the tissues.

As has been stated, the molecular weights of these extracellular pigments are considerable and, from this fact, the osmotic pressure which they exert is extremely small, which seems to correspond to a physiological necessity. In fact, if present in the same medium in the form of smaller molecules they would exercise an osmotic pressure incompatible with their physiological participation in the osmotic exchanges. In a parallel study of the respiratory capacity of the blood of various animals, containing the transporters of oxygen dissolved in the plasma, in relation to osmotic pressure, it has been shown

by Florkin (41) that only the respiratory pigments having the large molecules can exist in a sufficient quantity to assure oxygenation without disturbing osmotic relationships at the level of the vessels. Nevertheless, the feeble pressure exerted by these bodies ought to influence osmotic exchange mechanisms to the same extent as the "oncotic pressure" of mammalian plasma.

The physico-chemical study of the hemocyanins brings to light a new aspect of the problem of the specificity of the respiratory pigments seemingly limited, heretofore, to the invertebrates, and in relation to the regulation of osmotic exchange by the blood proteins.

Two observations are at the basis of this new order of facts. On the one hand Svedberg established (106) that the hemolymph of *Octopus vulgaris*, of *Limulus polyphemus*, and of other species contains hemocyanin in different micellar states, some probably capable of giving rise to the others, reversibly. This is a fact which seems to be sufficiently frequent in the invertebrates to have a general significance. On the other hand, Roche & Roche (87) have shown that the same pigment is capable of existing in various species of *Helix* in particles of very different dimensions according to the living conditions of the animal. In the course of hibernation in *Helix pomatia* and of estivation in *Helix pisana*, the particle size of the chromoprotein present in the hemolymph may vary from that of the simple to the double form.

It is not certain that these facts, last-mentioned, necessarily mean polymerization and depolymerization of the same particles. On the contrary, in this particular case, we think that the chromoprotein renews itself rather rapidly in the hemolymph and that it retains the same size during all its sojourn in this medium. If this is so there should be formed molecules of hemocyanin of various dimensions according to the different physiological states (hibernation, prolonged fasting, estivation). The successive formation of micelles of various sizes and the displacement of the equilibrium in a heterogeneous mixture of particles can, moreover, take place reciprocally. It would obviously be of interest to find out the influence of the physico-chemical state on the affinity of the respiratory chromoproteins for oxygen. Unfortunately such researches have not yet been undertaken.

The possibility of the existence of the same pigment in various micellar states, simultaneous or successive, extends the problem of the specificity of these substances outside of its usual limits. In fact, these observations lead us to recognize that there may exist not only

a *molecular* specificity which extends to the composition, to the structure, and to the properties directly referable to these (ultraviolet spectrum, isoelectric point, ionic valence), but also a *micellar* specificity in which the size of the protein particle is the principal characteristic. The co-ordination of these factors of individuality, bearing upon one or the other, determines the *physiological* specificity of each pigment.

Remarks on the comparisons in composition of the four pigment types.—Our eagerness to find analogies in the constitution of these pigments sufficient to explain their common property of transporting oxygen, has often caused us to group them together without just cause. Actually it is possible to prepare the various respiratory chromoproteins in the pure state (Dhéré; Florkin; Fox & Roche; Roche), to make precise measurements on their elementary constitution and amino acid composition, and to draw conclusions with respect to their prosthetic groups. It seems to us certain that all the efforts at comparison, made heretofore in attempts to find a similarity in the constitution of these substances, have failed, and that they have been pursued in a field where there is no longer any profit in continuance.

We are still almost entirely ignorant of the nature of the linkage of oxygen to the respiratory pigments, and nothing permits us to foresee how they combine. It is certain that the metal participates since it exists in a constant proportion with the oxygen. But the protein part plays an equally important rôle, since it is only when the hematin is combined to a globin that it can form with oxygen a dissociable compound. Now, the prosthetic groups of the respiratory pigments are different (protoheme, chlorocruoroheme, hemocuprin, hemoferrin), the protein constituents the same. Moreover, among the protohematin pigments (hemoglobin and erythrocrucorin) one finds some very different proteins. And yet it is probable that the respiratory property devolving upon these substances, even though different, ought to have the same basis, composed of an ensemble of active groups. This is a problem which remains for solution. For the moment it is hard to imagine how it could be attacked.

HEMATINIC NON-RESPIRATORY PIGMENTS AND DERIVATIVES

Cytochromes and the respiratory ferment.—The most striking point in the development of the question of cytochrome is the increase of its complexity and its similarity to the "enzymic transporter of oxygen" or "respiratory ferment" of Warburg with cytochromes.

From the beginning of his research in 1925 Keilin had observed that the relative intensities of the four bands (*a*, *b*, *c*, *d*) of the typical spectrum of cytochrome are variable, according to the tissue examined. More recently spectra of cytochrome have been described in which one or more of these bands are missing or noticeably displaced. Fink (36), Elion (33), and Urban (112) observed in brewers' yeast a spectrum where *b* and *c* had fused, and *a* was very feeble and displaced. Similar results were obtained by Roche on various actinians (91). The unique band (*b* + *c*) occupies a position very close to *b*, while *a* is noticeably less removed from *b* than in the classical spectrum. Likewise with *B. coli communis* [Keilin; Fujita & Kodama (45)] the *c* band is missing and *a* occupies the same position as in the spectra of the actinians. These organisms then do not contain cytochrome-*c*, in quantities at all abundant.

On the other hand, one observes in the spectra of *B. coli communis*, of *Azotobacter*, and *B. aceticum* [Warburg & Haas (114), Negelein & Gorischer (77), Tamijia & Tanaka (109), Yamagutchi (120)] an absorption band at 635 mμ, non-existent in the classical spectrum of cytochrome. As this band, *a*₂, has been found only in organisms where the *a* band is missing, one agrees with Keilin (63) and Shibata (10) that it appears to be a derivative of cytochrome-*a*. But while the other bands (*a*, *b*, *c*, *d*, and *a*₁) always maintain the same positions in the spectrum of an organism or of a tissue, appearing and disappearing simultaneously or successively, it is not the same for *a*₂. This occupies in anaërobiosis a position which differs by several angstrom units depending on whether or not the medium contains potassium cyanide or carbon monoxide. This fact suggests to Warburg & Haas (114) that the band *a*₂ belongs to a compound which, contrary to the others (chemically more inert), combines with carbon monoxide and potassium cyanide, i.e., to the respiratory ferment. Keilin and Shibata do not admit this point of view. To them, the band *a*₂, as also *a*₁, pertains to a hematin which is derived from the compound with band *a* and replaces it. These observations show, incidentally, that organisms exist in which certain elements of the typical cytochrome may be missing, and that, on the whole, the intensity of respiration (aërobic) appears to be proportional to the richness in cytochrome *b* + *c*.

Certain flagellates synthesize with great facility the "respiratory ferment," starting out from protohematin [Lwoff (74, 75)].

The nature of these substances remains obscure. One considers them as protein parahematin. Only cytochrome-*c* has been isolated

in a state of purity sufficient for analysis [from yeast by Zeile (122) and from heart muscle by Theorell (110a)]. It contains 3.4 per cent of hematin; like the hemoglobins, its protein constituent has an isoelectric point close to that of the globins (Zeile) or more alkaline (Theorell), the identity of the products obtained by the two authors being doubtful. The constitution of its prosthetic group⁴ is incompletely known. Its heme is very probably a condensation product of protohematin 1X (of blood) with a nitrogenous base (Zeile). According to Keilin (64), each cytochrome, *a*, *b*, and *c*, contains a particular porphyrin, but according to Roche (91) the hemes of cytochromes-*b* and -*c* would be either identical or differing only in the nitrogenous base fixed to a side chain of the porphyrin nucleus.

Bigwood, Thomas & Wolfers (20) find that the purified oxidized form of cytochrome-*c* presents two bands in the red, the one occupying the position of that of Warburg's respiratory ferment; after prolonged dialysis the reduced form of cytochrome-*c* becomes autooxidizable (20).

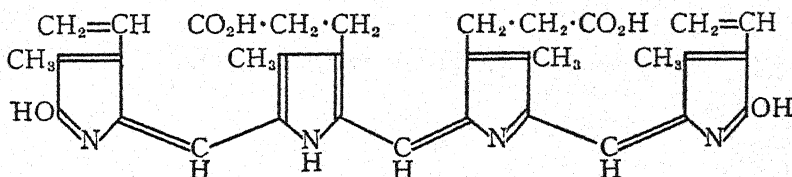
Other hematinic derivatives.—Roche (91) has been able to relate MacMunn's actiniohematin to the cytochromes and to Keilin's "free intracellular hematin" (protein-containing parahematin). It is a mixture of pigments composed principally of cytochrome-*b* and a parahematin presenting the characteristics of the "free intracellular hematin" (protohematin) of Keilin. It is not permissible, therefore, to consider actiniohematin as a pure compound analogous to heli-corubin.

In connection with the enzymes and the hematinic prosthetic group see reviews by Treibs (11) and Reid (7). Stern has shown that hepatic catalase contains a hematin derived from aetioporphyrin III, thus possessing the same porphyrin skeleton as blood protohematin (104). This enzyme forms with peroxides labile combinations comparable to those of the methemoglobins with peroxides (Stern). The liver catalase may be separated by dialysis into two constituents, one hematinic and diffusible, and the other non-diffusible—probably a protein. Each fraction is inactive but the mixture possesses catalytic activity [Agner (16)].

Bile pigments.—The study of the constitution of these compounds and their derivatives has been carried out principally by Fischer & Haberland (37), Fischer & Hartmann (39), Fischer, Halbach &

⁴ Cf. *Ann. Rev. Biochem.*, 3, 410 (1934).

Stern (38),⁵ and Lemberg (70, 71). The work of Lemberg has a particular interest for biochemists because it pertains to the transformation of hemins into pigments of the bile type. The oocyan of egg shells and the uteroverdin of dog placenta are identical with dehydrobilirubin prepared by the action of ferric chloride on natural bilirubin (70). The study of these compounds and of their degradation products and various other arguments lead to the suggestion that bilirubin is formed from hemin through biliverdin (I) and that bilirubin contains vinyl groups, as in biliverdin and protoheme.



I. Biliverdin

In addition to oocyan, the shells of various eggs contain, as a precursor of this pigment, some protoporphyrin [Dh  r   (30) and Gouzon (49)], the mechanism of opening the porphyrin nucleus being complex. After having shown that the artificial "green hemin" of Warburg & Negelein (obtained by oxidation of protohemin) is a derivative capable of giving "verdohemochromogens" and containing the nucleus of isobiliverdin (and not that of a porphyrin) Lemberg concludes that the formation of a verdoheme is perhaps the first step in the production of the bile pigments in the animals on oxidation of protoheme. Biliverdin is first formed, then bilirubin (71).

Important work on the biochemistry of urobilin is due to Watson (115, 116, 117), who has definitely demonstrated the identity of urobilin and of stercobilin and has obtained, quantitatively, mesobilirubinogen by reduction of the natural products.

Flavins.—These pigments have been reviewed by Kuhn in volume IV⁶ and are discussed elsewhere in the present volume.⁷ We call attention also to other reviews by Kuhn (4) and Wagner-Jauregg (12).

⁵ Cf. *Ann. Rev. Biochem.*, 3, 410 (1934).

⁶ *Ann. Rev. Biochem.*, 4, 479 (1935)

⁷ Cf. pages 8, 33, 189, 359. (EDITOR.)

Carotenoids.—One finds in animals carotenoids of two kinds, those of plant origin and those of animal origin. The former (xanthophylls and others) have been studied in connection with the transformation of carotenes into vitamin A and the assimilation of the xanthophylls and lycopin.

The most extensive papers on the second subject are due to Zechmeister (13), in particular on human fat, from which has been isolated a mixture of compounds containing lycopin, α - and β -carotene, capsanthin, and xanthophylls. There exist, in this regard, differences among the depot fats of various animals (chicken, horse, pig) because they absorb differently the various carotenoids [Zechmeister & Tuszon (121)]. The serum carotenoids are likewise of plant origin [Verzar, Süllmann & Vischer (113)]. Various other carotenoids, some not well known, have been found in the tissues or cutaneous structures of animals in which they are formed, often at the expense of plant carotenoids previously digested. Such is true of pigments of canary feathers. If these birds receive a diet lacking in carotenoids, their feathers are seen to bleach, becoming yellow again when given xanthophylls from which the organism produces a pigment (canary-xanthophyll) similar to taraxanthin [Brockmann & Völker (23)].

Various carotenoids in actinians have been studied by Fabre & Lederer (36) and Heilbron, Pary & Jones (58). Astacin has been shown to be present in different Crustacea [Fabre & Lederer (35)], in the muscle of salmon [Sörensen (103)], in some copepods of plankton [Euler, Hellström, & Klusmann (34)], and in the shrimp, *Palaemonetes*, by Brown (24). Various other carotenoids have been isolated by Lederer (69) from the tegument of *Pecten*, of insects, and of Ascidia. These problems have been reviewed by Lederer (5).

Other pigments.—Various other pigments known from old preliminary observations have been studied recently in more precise fashion. Let us mention here bonellin of *Bonnellia viridis* [Dhéré & Fontaine (31)] which is probably a porphyrin, the tegumentary porphyrin of *Lumbricus terrestris* [Dhéré (30)], and the blue pigment of the serum of the Labridae which is considered to be a phycocyanin [chromoprotein of the blue algae with a prosthetic group of the bile-pigment type] Fontaine (42).

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LABORATOIRE DE CHIMIE BIOLOGIQUE
FACULTÉ DE MÉDECINE ET DE PHARMACIE
MARSEILLE, FRANCE

METABOLISM OF CARBOHYDRATES AND ORGANIC ACIDS IN PLANTS

(EXCLUSIVE OF BACTERIA AND FUNGI)*

BY WILHELM RUHLAND AND JOHANNES WOLF

University of Leipzig, Germany

Methods for determination of carbohydrates in plants.—Since our last report¹ not very much new material has been added. Boiling in water (105) or in a strong alcoholic solution, which has been made slightly alkaline with ammonia (79), serves to extract the material. If one employs a copper-reduction test it is better to replace the Bertand method by that of Schoorl (179). The usual addition of calcium carbonate in the extraction with water is detrimental according to Denny (57) because it disturbs the analysis of different constituents when present in excess. Therefore special conditions are given for the preparation of tissues, in the absence of calcium carbonate, without danger of inverting sucrose and inulin. The clarification of the extracts is accomplished with basic or, occasionally, with neutral lead acetate, with mercuric sulfate (203) or also with aluminum hydroxide (207). In the case of plants, however, the non-sugar reducing substances are never completely removed in this process, and, on the other hand, there is danger of losing some of the sugar. Therefore charcoal is used to better advantage for the removal of color, which is indispensable for the titration according to Hagedorn-Jensen. This charcoal serves at the same time as an agent for the separation of the different kinds of sugars. In accordance with this Kerstan (105) has worked out a system of adsorptions and elutions, which permits the determination of the essential hexoses, including the glucosides and maltose, in the plant extract. The fundamental shortcomings of the reduction methods, which are due to lack of specificity, are corrected by such adsorption processes, but also to a certain extent by use of yeast fermentation.

Recently the Barcroft manometer has been used for the micro-determination of carbon dioxide produced by fermentation (156). Particular importance might be attached to the use of the cell-free

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juice, pressed from yeast, recommended by Malyoth & Sommerfeld (132). With this a 100 per cent glucose fermentation without disturbing side reactions (use of sugar for the structural metabolism of yeast and for by-products) and without respiration was attained. Addition of acid potassium phosphate accelerates the fermentation. A modified Krogh microrespirometer is suggested to measure the carbon dioxide.

Enser (64) gives technical improvements for the determination of starch in plant tissues. Denny (58) has made a comparative study of several methods which serve the same purpose. The one consists in digesting the powdered tissue with takadiastase at pH 4.5 for forty-four hours followed by determination of the increase in copper-reducing power in the absence of acid hydrolysis. Another method depends upon the extraction of starch with hot concentrated calcium chloride solution, in which starch is soluble. This method usually gave lower starch values, so that presumably the takadiastase which is used in the first method attacks other polysaccharides (hemicelluloses). A third method by the same author (59) follows very closely the method of Rask (170), in which the material is ground with cold hydrochloric or sulphuric acid and extracted. The starch is precipitated with alcohol and then hydrolyzed.

OCCURRENCE AND CHEMICAL RELATIONS OF PARTICULAR CARBOHYDRATES

Sugars.—Trehalose was found in *Rivularia bullata* (Cyanophyceae) in the absence of sucrose and reducing sugars (50). Synanthrin, a fructosan of the Jerusalem artichoke, discovered by Tanret (1893), contains glucose as a structural unit [Colin & Chaudun (48)]. Its molecular weight is supposed to be about 900 which would indicate a complex formation of 5 or 6 anhydro-glucose residues. According to Colin & Belval (44) raffinose occurs in the germ of wheat, rye, barley, and oats, but not in that of corn or millet. In the endosperm the amount of levosin and sucrose decreases from the outside towards the center (72). Levosin, which according to Colin & Belval (44) is not present in wheat germ, is found in the stalks (45). In the seed grains from *Coronilla* are found the well-known stachyose of legumes and saccharose (196). In unripe pumpkins Tichmenew (200) found a small amount of glucose and considerable *d*-fructose. There are several important contributions which deal with the

steric conversion of the individual hexoses into each other. That such a conversion must occur in metabolism has recently been shown again by Virtanen & Nordlund (204) who observed sucrose formation in starch-free leaves which were nourished with glucose or fructose. Nelson & Auchincloss (140) come to the same conclusion by using potato slices as experimental material. According to Lohmann's (125) observations it is probable that a phosphorylation of the sugar precedes such a conversion for it could be shown that the Neuberg ester, as well as the aldose-monophosphoric-acid ester, is converted by the organism into the Embden ester (equilibrium mixture of the aldose- and ketose-monophosphoric-acid esters. Of importance in this connection is the discovery by Burkhard & Neuberg (27) that a mixture of fructose- and glucose-phosphoric-acid esters occurs in the leaves of the sugar beet. If it is assumed that the enol form of the sugar takes part in such a conversion, then galactose, which behaves differently in this case from the enol form common to the three other zymohexoses (glucose, fructose, and mannose), must occupy a special position. Through their investigations of germinating seeds of *Trigonella foenumgraecum*, Daoud & Tadros (55) come to the conclusion that in this isomeric change of one hexose sugar into another in the living organism the formation of an intermediate polysaccharide or a condensation product plays an important part. It is suggested that the actual transformation of the sugar takes place on polymerisation or condensation in one of two ways: either a reversal of the stereochemical configuration of the carbon atom concerned in the linkage of pairs of the hexose residues, or an intramolecular rearrangement between the oxide ring and a hydrogen atom on the first and second carbon atoms of the hexose molecule. The oxide ring moves to the carbon atom concerned in the linkage of pairs of residues. The former case occurs in the transformation of a sugar into its stereoisomeride (e.g., galactose \rightleftharpoons glucose) and the latter case during the transformation of a sugar into its ketose analogue (glucose or mannose \rightleftharpoons fructose).

Sugar alcohols.—Spoehr and others (186) and Reif (172) report concerning the occurrence of *d*-sorbitol in fruits of the Rosaceae. In *Photinia arbutifolia* Linn. this alcohol not only occurs in the fruit [toyon berries (189)] but also in the leaves (42). As frequently in the Oleaceae, mannitol occurs in the leaves of *Nyctanthes arborescens* which belongs to this group of plants (121). Braecke's (16) discovery of mannitol in *Pedicularis* (Scrophulariaceae) as well as in

unripe olives (147) is to be regarded in a similar way. Hassid (85) found dulcitol in *Iridaea laminarioides* (red algae) and thereby confirmed the statement of Haas & Hill. Hassid assumes that an equilibrium exists between the galactan and the dulcitol, analogous to that between starch and glucose in higher plants.

Starch.—Colin (43) investigated the starch of the Florideae and found that it differs in many respects from potato starch. This author considers it to be a kind of erythrodextrin, or as an intermediary between starch and glycogen with special proximity to the latter. The wood starch of certain deciduous trees (oak, walnut) is, according to Campbell (35), an acid polysaccharide 90 per cent of which consists of anhydro-glucose units, whereas the remainder is apparently made up of a mixed anhydride of glucose and glucuronic acid, which is perhaps partially methylated. The question arises as to whether we are here really concerned with a homogeneous substance in the chemical sense, or whether the uronic acid is to be considered as an impurity, for Niemann, Roberts & Link (142) were able to extract a starch polysaccharide from the woody tissue of ripe apple twigs, which was identical in structure with the β -amylose of cereals and potatoes. On the other hand it is known that the insoluble α -amylose shows a tendency towards esterification and moreover that in the seeds of cereals it is esterified with palmitic, oleic, and linolic acids. Taylor & Sherman (197), who were able to split up this ester by the action of acids, bases, and lipase-free amylase, find that the linkage between the unsaturated part of the acids and the carbohydrate is less stable than the linkage between the saturated part and the carbohydrate.

Hemicelluloses.—The investigation of this group suffers from "an unfortunate lack of conciseness in the application of the term 'hemicellulose,' which has been adopted to include a widely differing collection of substances with often only certain physical properties in common" (29). It is also very doubtful whether the fractions obtained by the method of Preece, which is the one hitherto most extensively used, are uniform and represent a definite substance. Buston (29) suggests

a more satisfactory basis of classification, if it could be applied, would recognise the following types:

I. compounds predominantly hexosans.

II. intermediate compounds comprising hexosan-uronides, pentosan-uronides, etc.; these might be of any degree of oxidation, from the but slightly

oxidised compounds of the type isolated from lichens by Buston & Chambers (31) to the more complex bodies in which a considerable amount of pentosan formation has occurred.

III. compounds predominantly pentosan, such as the xylans of maize-cobs, straw, etc.

Further, examples of each of the three types could arise from glucose, galactose or other parent hexoses. Thus pectin would become a particular member of type II of the galactose series; . . . and perhaps it is best to regard them (the pectins) as comprising a sharply defined group of highly oxidised galactarabans.

Peter, Thaler & Täufel (152) [cf. also Täufel & Thaler (191, 192)] consider the term "hemicelluloses" as purely historical and find it unnecessary on the basis of the following classification of the constituents of incrustated cell membrane:

- (a) Unsaturated part: lignin.
- (b) Saturated part:
 - (i) Glucose part (skeletal substance): cellulose; easily- and difficultly-soluble xylan.
 - (ii) Galactose part: galactan; araban; pectin.

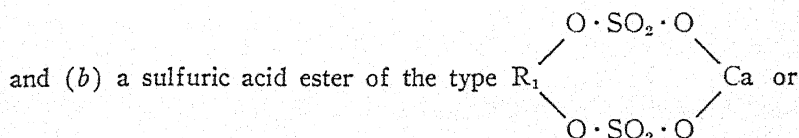
Hurd & Currie (96) isolated xylan from oat hulls, which after hydrolysis only gave *d*-xylose. The similarity which this material shows to different polyuronides induced Anderson & Krznarich (5) to verify the results. After the separation of the starch and pectin the extract obtained with a 5 per cent caustic soda solution contained a mixture of hemicelluloses. After hydrolysis of the hemicellulose the sugars *d*-xylose and *l*-arabinose and a compound of one molecule of *d*-glucuronic acid with two molecules of *d*-galactose were found. From analysis, the result of which depends upon the nature of the preparation, and from the behavior of the hemicellulose it follows that a mixture is present. This mixture may consist of a xylan and a polyuronide, which contains *d*-glucuronic acid combined with two molecules of *d*-galactose and a series of *l*-arabinose molecules, or two polyuronides may be present, as in oat straw (Norman, 1929). Votoček & Zvoniček (205) investigated the wings of a number of kinds of seeds, and always found xylose and lesser amounts of a hexose (galactose, mannose). Buston (30) investigated a series of hay grasses, in which the hemicellulose was separated into five fractions by Preece's method. The total hemicellulose (16 to 20 per cent of the dry weight) gave on hydrolysis a mixture of xylose, arabinose, galactose, and galacturonic acid.

Several authors are investigating the hemicellulose of trees. Thus Tottingham (201), who investigated the woods of apple, cherry, pear, peach, and plum trees, reports that the total hemicellulose content is 15 to 24 per cent glucose, and 37.9 to 81.3 per cent xylose; the uronic acid content of plum wood was double that of other fruit trees. According to Otterson & Tottingham (150) the hemicellulose that is present in year-old apple twigs is primarily made up of glucosans, followed in importance by uronic acids and finally pentosans. Campbell (34) also finds that the hemicellulose of oak wood, which is closely related to hemicellulose-A, is composed for the most part of anhydro glucose (75 per cent); uronic anhydride (14 per cent) and anhydro xylose are formed only in relatively small quantities. Buston (29) advocates in general the view that the hemicellulose of woody tissues is characterized by xylan and allied hexopentosans of the same series (glucosan-glucuronicanhydride-xylan) while the hemicellulose of new woody tissues consists mainly of galacto arabans. This view is corroborated by the findings of O'Dwyer (149) to the effect that the wood-hemicellulose A consists mainly of xylan (82 to 85 per cent) and uronic acids (10 per cent). Uronic acid prevails in sapwood and methoxyaldobionic acid in hardwood. The latter decomposes on hydrolysis into xylose and partly methylated glucuronic acid. Worthly of mention as a special difference between sapwood and heartwood is the positive iodine reaction of the hemicellulose-A obtained from sapwood, so that the possibility exists that the hemicellulose-A molecule (insofar as this term is justified) contains a small percentage of anhydro glucose; glucose, on the other hand, could not be found among the products of hydrolysis. Campbell (34), who observed a similar color reaction with iodine in the cell walls of oak wood, assumes that the hemicellulose of oak wood is derived from the starch by oxidation of the primary hydroxyl group and the formation of anhydroxylose residues by decarboxylation. Spoehr and others (186) found two hemicelluloses in sapwood, which contain *d*-xylose and methoxyuronic acid.

Miyama (136) finds that the galacto araban in peanuts is made up of arabinose and galactose in the proportion of 2:1; a small amount of galactose is supposed to be oxidized to galacturonic acid. Klages (110) assumes that the 1:4 linkage of mannopyranose groups, such as he found in the mannan-A of the ivory nut, represents a general structural principle of the naturally-occurring high polymeric aldopolysaccharides. Daoud (54) had described the mannogalactan of

bock clover as a silico-phosphoric-acid ester. It was discovered by Iyer & Sastri (99) that the phosphorous content of the preparation was caused by an impurity and that accordingly no esterification of the mannogalactan by phosphoric acid occurs. Hassid (86) was able to find a further polysaccharide ester in the sodium salt of the sulfuric acid ester of the galactan in the red alga, *Iridaea laminarioides*.

A renewed study of agar by Takahashi & Shirahama (193) shows that two substances result on hydrolysis: (a) a simple polysaccharide



$R_1(\text{O} \cdot \text{SO}_2 \cdot \text{O})_n \text{M}$; from the second fraction kantenic acid could be separated which consists of galactose 39.47 per cent, pentose 8.17 per cent, and approximately 8 per cent of sulfuric acid. Uronic acids and methyl pentoses were absent. $R \cdot R_1 \cdot (\text{O} \cdot \text{SO}_2 \cdot \text{O})_n \text{M}$ might be considered as a general formula. By separating the two carbohydrate residues (R and R_1) the ability to gel is lost. This type of union is contrary to the view of Samec & Isajevic (1922). Butler's investigations (33) lead to the modified formula $[\text{R}(\text{O} \cdot \text{SO}_2 \cdot \text{O} \cdot \text{K})_{2 \text{ or } 3}]_n$ for the polysaccharide of *Chondrus crispus* which is in better agreement with the facts than the earlier one given by Haas. Butler (32), furthermore, observed the occurrence of nitrogen in the polysaccharide complex; the interpretation is difficult on the one hand because the proportion of the two components is not constant, on the other, because the nitrogen cannot be separated from the polysaccharide by repeated precipitation. Possibly the cause is either a simple adsorption upon the colloidal polysaccharide particles of a nitrogen compound of nature as yet unknown, or a "symplex" formation such as was found, above all, by Przylecki and his coworkers (157) between particular amino acids, lecithin, etc., on the one hand and polysaccharides on the other.

The seed mucilage of *Plantago psyllium* which was investigated by Anderson & Fireman (4) constitutes about 20 per cent of the dry weight, and is dependent as regards composition upon the method of preparation. It consists of a mixture of polyuronides which, according to the results of hydrolysis, is made up of *d*-galacturonic acid and *l*-arabinose, the latter being combined with a chain of 8 to 35 xylose molecules. In confirmation of Anderson & Crowder's (1930) reports

Niemann & Link (141), in flax-seed mucilage, find *d*-galacturonic acid, xylose, rhamnose, and *l*-galactose. The supposition that *l*-galactose, which has hitherto been found but seldom, may occur together with the corresponding *l*-galacturonic acid, which has not yet been found in nature at all, was not confirmed. The occurrence of an appreciable cellulose fraction in seed mucilage was demonstrated by Bailey (9) in cress seed, so that a more general significance may be ascribed to these findings. After hydrolysis the composition of the residual part of the mucilage was: *l*-arabinose, *d*-galactose, *l*-rhamnose, and *d*-galacturonic acid. Anderson (3) found two polyuronides in the mucilage from the bark of *Ulmus fulva*, which on hydrolysis gave galacturonic acid, *l*-rhamnose, *d*-galactose, and a colored, unknown compound X. A pentose, a methylated hexose, and a methylated uronic acid are probably also present. Concerning the structure of the polyuronides it has been reported that they are made up of a uronic acid, two simple sugars, and X. The pentose of the methylpentose combines with the aldehyde group of the uronic acid, which again combines with a hexose or a methylated hexose through its aldehyde group. X is bound by the aldehyde group of the hexose.

While the gum obtained from whole rye bread by a water extraction [Fellenberg (66)] represents a pure pentosan, Geoffrey (72) found arabans and glucosans in the gum of wheat flour. Of a more complex nature is lemon gum, in which Parisi (151) found 35.6 per cent of pentose (arabinose), 4.5 per cent of methylpentose, 19.2 per cent of galactose, and 18.1 per cent of a uronic acid which was not supposed to be identical with either glucuronic acid or galacturonic acid. According to Colin & Payen (50) the mucilaginous gum of the blue-green alga *Rivularia bullata* gives glucose, arabinose, and uronic acid on hydrolysis. A stimulating investigation by Weevers (206) is to be mentioned in this connection. The author sees in the gum production the formation, guided in other directions, of pectin materials and eventually of cellulose in young cells. Pectin may be considered as a special group of hemicelluloses because of its characteristic pectic acids. The recent enzymatic splitting of pectin to give *d*-galacturonic acid with pectolic acid as intermediate product, carried out by Ehrlich *et al.* (65), is a further proof that these substances are genuine decomposition products of pectin and not denatured products. Pectin cannot, however, be considered a completely uniform substance as is shown in the investigation of the sap- and heartwood of black locust by Spoehr *et al.* (186). These investigators

assume the existence of several pectic acids which differ according to the source of the material in the arrangement of the component *d*-galacturonic acid, hexose, and pentose. Colin & Chaudun (49) separated a mixture of pectate and araban from desugared beets by extraction with hot water. The successive extracts, however, differed so much in their composition that it is impossible to recognize in them the hydrolysis products of one and the same substance and to ascribe to the intercellular binding material (pectin) the composition of an insoluble complex of 80 per cent pectate and 20 per cent araban. Bridgham & King (26) find *d*-galacturonic acid, *l*-arabinose, *d*-galactose (ratio 4:1:1) and methyl alcohol in lemon albedo pectin. In the natural state the methyl alcohol is esterified with *d*-galacturonic acid.

In contrast to pectin it is possible to recognize a definite reserve-carbohydrate character in most of the other hemicelluloses and especially in such as contain hexoses. This is particularly evident in the fact that these substances are more readily drawn into metabolism than all other polysaccharides except starch. This conclusion is not affected by the fact that this substance may, through special deposition, serve other purposes, such as that of a supporting material in leaves (94), etc. The pectins, which in general are very stable in the tissues, therefore represent a building material to a much greater extent than the other hemicelluloses. Thus pectins form a much greater proportion of rapidly growing tissues (29); a high hemicellulose content in the tissues, like a high lignin content, seems to exclude an abundance of pectin. According to Kiesel & Jatzina (109) *d*-galacturonic acid, which is the chief component of pectin and forms 64.8 per cent of it, results from oxidation of carbohydrates in the cell interiors.

Cellulose.—The data hitherto available concerning the occurrence of cellulose in red and brown algae do not have a satisfactory analytical basis. Naylor & Russell-Wells (139) incontestably proved the presence of cellulose in four red and seven brown algae and investigated its distribution in the tissues. It was likewise possible for Dillon & O'Tuama (62, 63) to obtain cellulose from the Laminariae. This was made up entirely of glucose units. These investigations, as well as the x-ray diffraction patterns found by Khouvine (107) for *Equisetum*, *Hypnum*, and *Marchantia*, lend support to the idea of the similarity of the plant celluloses (yeasts and lichens excepted). According to Shikata & Watanabe (181) sphagnum cellulose has the

same structure as wood cellulose or cotton cellulose; slight differences in behavior toward sodium hydroxide were noted, however. Cellulose obtained from the cell walls of unripe pumpkins also give glucose on hydrolysis [Tichmenew (200)].

The investigation of "holocellulose" (which means the total carbohydrates in extractive-free tissue) of spruce by Kurth & Ritter (119) showed that a carbohydrate residue resembling that found by Cross & Bevan remains after hydrolysis with 1 per cent sulfuric acid; if a dilute acid is used an easily hydrolyzed hemicellulose fraction is obtained, similar to a fraction which by earlier analytical methods was considered a part of other wood constituents. It contains methoxyl-, carboxyl-, acetyl, and formyl-groups and gives mannose, galactose, glucose, arabinose, and xylose on hydrolysis. The fibers of the fruit of *Cocos nucifera* are known to be the best when the fruit is about ten months old. Menon (134) reports that at this time the cellulose content (method of Cross & Bevan) is at its minimum and the methoxyl content has reached its maximum. An increase in cellulose and a decrease in methoxyl content follows this minimum and maximum. Menon believes that this is caused by the transformation of lignin into tannin.

Lignin.—The probability of a genetic relation between lignin and polysaccharides is supported by Buston's (29) findings, who follows Klason's assumption that the main forerunners of lignin are the substances of the glucosan-xylan series. According to Hempel (87) the lignification itself first begins, in the sense of the Wislicenus theory of wood formation, after the formation of a cellulose skeleton upon which the lignin is deposited little by little; afterwards, through adsorption, coagulation, and storing, it is combined chemically and colloidally with the cellulose skeleton. According to Klason (111) there occurs in lignin a chain molecule (of molecular weight at least 3640), in which as a result of a continuous condensation principle coniferyl aldehyde complexes are bound together in the same manner as the simple sugars in the polysaccharides. Tetra-coniferylaldehyde seems to represent the highest step of the complex formation. Freudenberg (67), on the contrary, considers the lignin molecule to be a heteropolymeric molecule in which the binding principle varies within certain limits.

Still other groups of substances are found in lignified or otherwise transformed cell walls of older tissues along with pectin and cellulose. The important question, whether these groups are free

or are in some way united with one another or with the cellulose, has not yet been answered with unanimity. According to Lüdtke (126) no chemical bond exists between cellulose and lignin and between cellulose and xylan, but there does exist an organized community of growth among the main components (cellulose and xylan) in the free state. Freudenberg (68) also is of the opinion that cellulose and lignin are not chemically combined in wood. The impossibility of dissolving cellulose from wood with the usual solvents is caused, according to Freudenberg, by the fact that the cellulose swells and cannot diffuse out of the surrounding lignin tissue. Harris and co-workers (83) and Norman & Shrikhande (146) come, by different methods, to the conclusion that lignin can be chemically combined with the encrusting hemicelluloses or polyuronides. Such a combination between lignin and carbohydrates would then have the effect of stabilizing the natural lignin which occurs in the enol form and is therefore more reactive than the isolated lignin present in the keto form.

THE PHYSIOLOGICAL BEHAVIOR OF CARBOHYDRATES

The origin of carbohydrates and their transformation into one another and into other substances have been investigated in relation to the development of the plant (germination, seasonal rhythm, flowering and fruit, heredity, etc., internal factors), or their behavior was studied under special external conditions (mineral nutrition, temperature, water supply, diseases, etc.).

Internal factors.—Malhotra (127 to 130) carried out several investigations concerning the transformation of material during germination. Starch and hemicellulose, especially, are consumed in corn. Isolated endosperms without embryos only transform starch into sugar, while hemicelluloses, fats, etc., remain practically unchanged. According to Bourdoul (19) there exists a relation in various kinds of peas between the content of soluble carbohydrates and the swelling power at germination, during which period the starch decreases uniformly in the same proportion as the sugars increase. Sahasrabudde & Kibe have studied (177) the action of starch-hydrolysing enzymes during rice germination. It was found that the starch granules in older kernels were more easily attacked than those in young kernels (176).

Of great physiological interest are the continued investigations of Mason & Maskell (131) concerning the transport of material in

the cotton plant. These studies are concerned with the distribution of total sugar, of sucrose, and of reducing sugars. Since these investigations are, however, not of biochemical nature, they need be only briefly mentioned.

There were several investigations dealing with seasonal changes. Smyth (183) finds the maximum of monosaccharide in terminal shoots of apple trees at the end of January, and the minimum in April. The sucrose content, which is always small, fluctuates more strongly, being at a minimum in June and then increasing until autumn. The maximum occurs likewise in January. The sucrose is claimed to be a preliminary reserve material, while the hemicelluloses, which are particularly present in wood, serve rather as building materials. The amount of hemicellulose increases between June and August. There are two minima for starch, one in January and the other in June. There is a maximum in October and a smaller one in April. The greatest fluctuations of carbohydrate, including cellulose, occur when the buds swell and when the leaves fall. Particularly comprehensive balances of the seasonal economy for *Fagus silvatica* are reported by Gäumann (70). We will mention only the carbohydrates here. The consumption of materials caused by the winter respiration of the buds proceeds five-sixths at the expense of the hemicelluloses and one-sixth at that of the actual carbohydrates. In accordance with this the bud volume decreases at the rate of 1 mm. per day. From the middle of January on, carbohydrate production predominates over the consumption. When the buds begin to burst, there is a decomposition of hemicellulose and an exceedingly large carbohydrate change. A 110-year-old beech tree uses about 37 kg. of carbohydrates in the formation of its leaves; the tree supplies only about two-fifths while the remainder is supplied by the foliage, through its own assimilation. Beeches ready to flower had an available carbohydrate supply of about 115 kg., approximately two-thirds being in the trunk. About 45 kg. of carbohydrates are removed from the tree by flower and fruit formation. One-half of this amount is hemicellulose and all parts of the tree, even the roots, contribute to it, the branch wood giving the most, i.e., about 87 per cent of their entire supply. In the falling leaves only 7 to 8 per cent of the former content can be found. Less comprehensive are some reports by Knowles, Watkin & Hendry (112) concerning the much-investigated sugar beet. On the other hand the work of Haas, Hill & Karstens (82) concerning the seasonal metabolism of *Corallina officinalis*,

a calcareous marine algae, is worthy of much greater attention because practically nothing is known about the metabolism of these plants. Only floridoside, a galactoside of glycerine, whose presence in the alga has been shown by Haas & Hill (80), can be considered here. The content of this substance is the greatest at the end of May and the smallest at the end of January. The fact that during the active life period of the alga the curves for the floridoside content and the amino nitrogen are reciprocal suggests the working hypothesis that the constant presence of peptides in these marine algae, which has already been proved, is due to a lack of balance between the metabolism of carbon and nitrogen (81).

According to Klebs the carbohydrate content of higher plants is significant for the physiology of development in so far as a maximum value of the ratio of this content to that of the nitrogenous substances represents the preliminary condition necessary for the transition from the vegetative to the flowering state. Maume & Dulac (133) have investigated the dependence of this ratio upon climatic conditions in wheat. Hurd-Karrer & Dickson (97), also studying young wheat plants, found a considerable influence of the photo-periodicity upon the carbohydrate-nitrogen ratios. It was the greatest on long days, which accelerate flowering, and the smallest on short days, when flowering is retarded. Other external factors, particularly temperature, were investigated as to their effects. On the other hand Purvis (161) does not recognize differences in the nitrogen and sugar content as decisive for vegetative or reproductive behavior. For certain winter cereals, to be sure, she finds that "an increase in reducing sugars was apparent just before flower emergence, but since this was preceded by flower differentiation, it may be regarded as a result rather than a cause of the onset of the reproductive phase."

Maturity completes the year's cycle. In the ripening currant, according to Sobolewska & Turetzka (187), sugar flows out of the leaves. The transformation of the sugar in the berries is represented by the following scheme: sucrose \rightarrow (glucose + fructose) \rightarrow polysaccharide \rightarrow glucose + fructose. The accumulation of sucrose at the end of the ripening period would therefore be an interruption, not of the synthesis of glucose and fructose, but rather of the splitting process. The formation of organic acids is connected with carbohydrate metabolism. A few other reports on the behavior of carbohydrates during the ripening period may be briefly pointed out: thus that of Nishida (145) on the sosetsu seed of the "Sosetsu," a Japa-

nese sago; of Thor & Smith (198) on the hickory nut (*Carya oliviformis*), and of Platenius (154) on carrots.

Information has hitherto been scanty regarding sexual differences in plant metabolism. Differences in soluble carbohydrate metabolism between male and female individuals have been found in several cases of dioecious plants. Bouillenne, Bouillenne & Ghenne (18) report concerning *Mercurialis perennis* that at the beginning of the vegetation period the male beet leaves contain more than twice as much carbohydrate as the female. The consumption of soluble carbohydrates during the formation and anthesis of the flowers is considerably greater in the male plants, so that at the time of flowering the female plants are richer in sugar. The soluble carbohydrates consist essentially of sucrose and maltose (17). Talley (194) has found similar sexual differences in hemp during the time of flower development. On the average the male plants contained more total carbohydrate, polysaccharides, and sugar than the female. Especially great was the superiority of the male plant in reducing sugars, whereas the female was richer in nitrogen.

Concerning the heredity character of the ratio of different carbohydrates to each other, Bourdouil (20) found that "smooth" and "wrinkled" peas differ in the ratio, p , of starch to sugar in the ripe seeds, the ratio being greater for the former. In the hybrids not only the smooth seed dominates, but also the corresponding ratio, p , characteristic of the smooth type, whereas the pollen of the wrinkled peas in the pods of the smooth variety does not exercise any influence upon the synthesis of starch in comparison to the seed of the mother plant. Of particular interest for breeding appear to be the investigations of Arasimowitsch (7) who found that the chemical characters are to a large extent of polymeric nature and that one can arrive at conclusions only by studying the heredity of each individual substance, such as the individual sugars, but not by studying the mere totality, "sugar." The author has investigated several kinds of Asiatic water melons and their hybrids for glucose, fructose, and sucrose, and found that each sugar is separately inherited according to Mendelian law (*mendelt*). Glucose especially, but also fructose, showed themselves generally to be polymer-dominant, while sucrose behaved as a polymer-recessive character. Cyclic-reciprocal crossings are recommended for further investigation. On the other hand the parental content with respect to different materials appears to be able to prevent crossing. Colin & Charles (46) have shown that the types

of iris which they have investigated may be divided into three groups according to their carbohydrate content, such that the members of one group will not cross with those of another: (a) *Iris germanica*; with starch, without fructosan. (b) *I. pseudacorus*; irisin without starch. (c) *I. foetidissima*; starch and two fructosans not identical with irisin.

Among the biogenetic relations of carbohydrates to other groups of materials the relationships to the fats are of great physiological and chemical interest. Formerly it was generally assumed that with the coming of winter a far-reaching transformation of starch into fat, which reversed in spring, occurred in numerous woody plants; Ishibe (98) was unable to find such transformations in the seven types of plants (among these, two evergreens) he investigated. On the contrary, it was found that the curves of both groups run to a large extent independently of each other. It is regrettable that the author did not consider the soluble carbohydrates at all.

On the other hand it is not to be doubted that in woody plants fat may be transformed into carbohydrates in the spring because analogous processes have been often studied during germination of seeds rich in fat. Such germinations have repeatedly been made the basis of efforts to explain the as yet unknown chemism of the transformation in question. Since fats are poorer in oxygen than carbohydrates, smaller respiratory quotients result on burning. For the germinating castor bean both Murlin (137) and Daggs & Halcro-Wardlaw (53) find in this manner that the seat of transformation is to be sought in the endosperm and not in the embryo or in the young plants. Pierce, Sheldon & Murlin (153), as a result of chemical analyses and comparative respiratory investigations, believe that 6 molecules of ricinoleic acid are oxidized to 2 molecules of sugar, 1 molecule of cellulose, and 3 molecules of carbon dioxide during the transformation. In the remaining portion of undetermined nature they suppose the oxidation product of a pentose to be present.

Zeller (211) has sought for intermediate products of the transformation of higher fatty acids into carbohydrates during germination of the fatty squash seeds. In agreement with the views developed by Euler concerning the synthesis of fats, the author found that the seeds which had been impregnated with a potassium sorbate solution showed a considerably increased starch value after two days. In autolysates of the seeds to which substances (sorbic acid, acetaldehyde, acetoacetic, pyruvic, and oxalic acids) had been added, which

may be regarded as possible intermediates in the transformation in question, the author believes he has observed a synthesis of starch instead of a hydrolysis. β -hydroxy-butyric, succinic, and lactic acids are supposed, in contrast to other substances, distinctly to repress starch decomposition in such autolysates according to the law of mass action. Therefore the author wishes to consider all such substances as intermediate products. According to him apparently no β -oxidation of the fatty acids occurs immediately in this transformation, as in animals, but takes place only after short carbon chains of 5 to 6 carbon atoms have already been formed.

Respiration and assimilation occupy the most prominent position among the physiological processes in which carbohydrates take part. From one of Kidd's (108) lectures on aërobic and anaërobic respiration of apples it may be stated that, according to experiments of the author and of Trout, a constant production of glycerine must take place. This does not accumulate, however, so that in the presence of oxygen it is probably resynthesized to sugar. Fructose (active) is considered to be the primary sugar of respiration. A more complete discussion may advantageously follow publication of the work. Dastur & Desai (56) have compared the aërobic and anaërobic carbon dioxide production of sprouting rice with the simultaneous loss of carbohydrates (as hexoses). It has been shown in both cases that the latter is much too small to explain the former. The authors believe it to be probable that the carbon dioxide excess results from oxidation of the organic acids which were formed in the protein synthesis. In judging these investigations the uncertainties involved are not to be forgotten, in particular the uncertainty regarding the quantitative determination of carbohydrates in plants. The opposite result was found by Archbold & Barter (8) in apples which were stored at 12° C. The loss of acid and sugar, expressed as carbon, was 17 to 30 per cent greater than that through loss of carbon dioxide.

The question concerning the type of assimilation of the produced sugar is still disputable. Kretowitsch (116) finds in the assimilating leaves of different plants at 10 a.m. more direct reducing sugars than crystalline polysaccharides and especially more crystalline fructosides. In this case there was more fructose than glucose.

External factors.—Most of the numerous investigations of the action of inorganic fertilizers upon the carbohydrate production of plants are carried out with an agricultural end in view, and only some of them are of physiological interest. For it remains doubtful whether

internal relations of fundamental importance exist, and, if so, their nature continues to be obscure. On the other hand the direct causes for differences in carbohydrate content cannot be discovered in such investigations. It is therefore not amazing that the results are often contradictory. Thus it is usually asserted that the chief function of potassium is connected with the synthesis of starch and its translocation. Street (190) also found that peas in water culture with an excess of potassium contain large amounts of starch and hemicellulose. Houghland & Schricker (95) on the other hand have determined in potatoes that the addition of potassium to the fertilizer caused a slight depression in the starch percentage. Böning & Böning-Seubert (12) believe they have found definite dependence of the sugar content of tobacco leaves upon the mineral nutrition. These results harmonize just as little with those of Street (190) on peas as with those of Hartt (84) on sugar cane. Under various light exposures Street found the greatest sugar content when the plants were nourished with solutions rich in magnesium. In calcium nitrate solutions less total carbohydrate was found than in all other solutions. Hartt found that with potassium deficiency there were more reducing sugars and less cane sugar in the sugar-cane leaves, whereas the percentages of total sugar remained about the same. Potassium deficiency had a similar effect upon the stems. If the latter was especially large, the amount of total sugar decreased greatly. Potassium deficiency is supposed to disturb the transformation between hexoses and sucrose as also their translocation.

Further investigations concern the dependence of carbohydrate metabolism upon temperature. In the leaves of *Viburnum dentatum* and *Syringa vulgaris*, Denny (60) found that before the first frost there were no important changes in total carbohydrate, in contrast to the nitrogenous substances which were lost. According to Constantinescu (51) the amount of total sugar in young winter barley increases when the temperature falls. The differences in sugar content between varieties of differing resistance to cold are, however, only small and variable. Fuchs (69) has investigated the contribution of sugar to the osmotic value in wheat. Böning (12) has made similar investigations with tobacco. Since sugar is supposed to have a stabilizing action upon colloids, the resistance to cold should run parallel to the sugar content and to that portion of the osmotic value referable to sugar. The relationships between sugar content and resistance to cold in rye have been thoroughly and critically illuminated by Åkerman, Andersson

& Lindberg (2). As regards higher temperatures, Nightingale (144) has investigated the effect of temperature between 13° and 35° C. upon various metabolites, among others the carbohydrates, of the tomato plant. At 13° C. the accumulation, especially that of starch, was very great, whereas at 35° C. the plants upon feeding with nitrate consumed a particularly large amount of carbohydrates in protein formation and in respiration, and died earlier than those without nitrate addition. Nightingale (143) also carried out similar comprehensive investigations concerning the effect of temperature upon the roots of apple and peach trees. Here too the carbohydrate content was much greater at lower temperatures.

Hardening against drought is effected by causing the plant to suffer the limits of water deficiency. Resistance to frost like that to drought is frequently regarded as largely determined by carbohydrate behavior. Vasiljev & Vasiljeva (202) showed that in wheat, immediately after wilting, the monosaccharides and sucrose increase, and the hemicelluloses decrease somewhat. After more extended wilting the monosaccharides increased further, likewise the hemicelluloses, but the sucrose decreased. Immediately after watering the monosaccharides decreased again, likewise the sucrose, but hemicelluloses increased. Eight days later, after the plants were recovered, sucrose and hemicelluloses increased again at the expense of the monosaccharides.

In conclusion, the carbon dioxide content of the air may be mentioned among the conditions influencing the metabolism of the carbohydrates. Thornton (199) showed that when potatoes were stored in an atmosphere of 30 to 60 per cent carbon dioxide, the respiration, as well as the content of sucrose and reducing sugars, were greatly increased over the controls.

GLUCOSIDES

The more general questions are dealt with, during the period here considered, only in sporadic works. Kerstan (106) to whom we are indebted for an analytical method for the determination of the sugar combined as glucosides in addition to the other carbohydrates in plants treats (105) the old and difficult question concerning the importance of glucosides in plants. His conclusion is that aesculin in no way represents a carbohydrate reserve as Pfeffer believed. Even when the carbohydrate deficiency is great it remains stable in the bark and is transmitted but poorly. The plants do not form a glucoside when

aglucone is added. Even a warm bath and narcotics do not effect a splitting of the glucoside by the plant. This occurs only at lethal concentrations of the narcotic, that is, post mortem. On the other hand the leaf glucoside takes part in the familiar daily fluctuations of the carbohydrate: decreasing at night and increasing during the day. Therefore its origin must be connected with assimilation. Even isolated leaves doubled their glucoside content in two and one-half hours when exposed to light. A nightly transport like that of carbohydrates could not be proved for certain. As Armstrong and Kostytschew had already supposed, the physiological significance is perhaps to be found in aglucone. This is detoxified through combination with sugar or is protected from oxidation and fixed at the point of origin in a difficultly permeable form. Guilliermond (77) has performed special cytological investigations concerning the origin of anthocyanin and hydroxyflavone within the plant cells and could not prove any genetic relationship with the tannins, with which they often appear associated.

Numerous papers which deal with the chemistry and occurrence of certain glucosides may be referred to as follows:

Phenolglucosides.—A glucoside of *Gnidia polycephala* which is similar to daphnin (175); salipurposide and naringoside (164); glucosides in *Salix repens* (166) and *Salix nigricans* (165), distribution of the glucosides (including one of the flavonols) among the Salicaceae (167), and in *Salix purpurea* (168a); salicoside (168, 169); philyroside (philyrin) and syringoside in *Philyrea* (114, 115); arbutin in *Vaccinium Vitis-Idaea* (71); primulaveroside in *Primula acaulis* (73); lusitanicoside in *Cerasus lusitanica* (88, 89) and a heteroside in the same plant (91); asebotoside and phlorhizoside in the bark of the tree Rosaceae as well as in the Ericaceae *Andromeda japonica* and *Kalmia latifolia* (25); buddleioside in *Buddleia* (209).

Flavones, flavonones, isoflavones.—Robinobiose and campherol-rhamnoside (212); robinoside in the blossoms of *Vinca minor* (162); buddleioflavonolside in *Buddleia* (208, 209); liquiritine in *Glycyrrhiza glabra* (182); a flavonol glucoside in *Arctostaphylos uva ursi* and in *Vaccinium* (138); butrine in the blossoms of *Butea frondosa* (122); sophoricoside in the fruit of *Sophora japonica* (38); genisteol (37); persicoside in *Persica vulgaris* (39, 40).

Anthocyanins.—Chrysanthemin in purple-husked maize (178); kuromamin which is identical with chrysanthemin (118); a cyanidin-3-5-diglucoside in Shiso leaves (*Perilla ocimodes* var. *crispa*) (117).

Anthracene glucosides.—Franguloside, frangularoside, emodin, etc., in the bark of *Bourdain* (21, 22, 23, 24); galicide in the roots of *Rubia peregrina* and *Galium verum* (93).

Cyanogenetic glucosides.—Occurrence in Nelson-meadow grasses [Gramineae, red clover, Swedish clover, lotus (174)]; in *Sorghum vulgare* (1); in *Glyceria spectabilis* and other grasses (75, 135); in *Molinia coerulea*, with data on seasonal variations (102, 103, 104); in *Melica* and *Gynerium* (74); distribution of amygdonitrilglucoside and amygdaloside in plants (155, 163); karakin in *Corynocarpus laevigata* (36).

Mustard-oil glucosides.—*d*-ribose as the sugar constituent of crotonoside (185).

Saponins.—Aglucone of primrose saponin (124); saponins from the leaves of *Hedera japonica* (120); a saponin from the soy bean (28); a new saponin and echinocystic acid (sapogenin) from *Echinocystis fabacea* (11); a sapotoxin from the tubers of *Xanthosoma atrovirens* (42); a sapogenin from *Styrax japonica* (184); tigogenin, a digitalis sapogenin, which is comparable to sarsapogenin from sarsaparilla roots (100).

Compounds with stearins.—Solanin-t and -s (148); stearin compounds of the glucoside type in soy-bean oil (101); a glucoside of γ -spinasterol in spinach fat (92); the identity of α -scillanic acid with allocholanolic acid (188).

Less known glucosides.—Asperuloside in *Coprosma Baueriana* (90); oleuropaein in olives (52); scillin in *Scilla maritima* (47); thevetin in the nuts of *Thevetia neriifolia* (41); verbenalin from *Verbena officinalis*, identical with cornin from the root bark of *Cornus florida* (171); coronillin from *Coronilla varia* (195); bryonin from *Bryonia dioeca* (6).

ORGANIC ACIDS

By way of introduction we note the important and successful investigations of Pucher, Vickery & Wakeman (158, 159, 160) concerning the determination of organic acids (malic, oxalic, and citric acids) in plant tissue. Kotnitzki & Bogatirtschuk (113) give a simplified method for determining citric acid.

Dillon & McGuinness (61) find that alginic acid, a uronic acid, is combined with colloidal iron and calcium components in the living cell (for example in *Laminaria*) and that it has the formula $C_6H_8O_6$.

In the anhydrous state it forms a lactone. Lebedew & Lindequist (123) found quinic acid along with citric and other acids in the moss berry.

With the aid of Thunberg's enzymatic chemical method Borgström (15) succeeded in demonstrating the presence of citric acid in the leaves of several species of *Kleinia* (succulent Compositae), in some cases in considerable amounts (up to 16.7 per cent of the dry weight in *Kleinia nerifolia*). No citric acid was found in the Crassulaceae (14), but an interesting physiological parallel exists in that the acid content decreases during the day in *Kleinia*. Borgström therefore interprets these varieties as new types of succulent plants (citric acid plants). Since Guthrie (78), moreover, reports that citric acid is present in Crassulaceae (*Bryophyllum calycinum*), and that it takes part in the fluctuations of the total acidity to the extent of 25 per cent, the two families may possibly differ only in a preferential participation of the one or the other of the two acids in metabolism. Ricevuto (173) finds that citric acid is formed in lemons during the months of maximum temperature (August, September) at the expense of the reducing sugars and of the pentosans; later (October to March) the pentosans alone are supposed to supply the material. The author advances the hazardous hypothesis that the formation of the citric acid is the result of an enzyme which is supplied by an *Aspergillus* existing in the surrounding soil.

Bohn (13) attempted to discover the rôle played by oxalic acid in plants. He finds that the amount of oxalate in the hyacinth is the same in the bulbs at rest as in the mature plant. It was immaterial whether these were stored in darkness or in the light. The author, as yet, has given no answer to the important question as to whether during development the oxalate is dissolved in the bulbs and later re-formed. Zacharowa (210) studied among other things the behavior of oxalic and malic acid in the needles of *Picea excelsa* during the winter. According to Zacharowa these acids are to be considered neither as the products of carbon dioxide assimilation nor as decomposition products of proteins or of amino acids, but rather as resulting from incomplete oxidation of carbohydrates. This paper, which is rich in hypothetical assertions, has in our opinion not been able to prove that a connection exists between the malic and oxalic acid content and the "oxidation conditions." Guillaume & Léo (76) investigated the variations of acidity in the ripening of huckleberries and found that the citric acid content decreases in this process (1.1 per cent acid in the half-ripe berries and only 0.832 per cent in the ripe ones). The malic acid

content increases during this time from 0.040 per cent to 0.100 per cent. Shibata, who investigated the relations between organic acids and nitrogen compounds in *Begonia Evansiana* (180), in general adheres to the idea of acid formation by deamination which is held by Ruhland & Wetzol. This is especially true for stems and leaf stalks. This type of acid formation, which Ruhland & Wetzol maintain to be characteristic also of the metabolism of rhubarb, is not recognized by Bennet-Clark & Woodruff (10) for these plants. These authors rather consider it probable that malic acid metabolism is intimately linked with that of carbohydrates, either in the processes of respiration or of photosynthesis.

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UNIVERSITY OF LEIPZIG
LEIPZIG, GERMANY

THE BIOCHEMISTRY OF THE NITROGENOUS CONSTITUENTS OF THE GREEN PLANTS*

BY GORDON T. NIGHTINGALE

*University of Hawaii,
Honolulu, Hawaii*

Most of the work on the nitrogenous constituents of green plants that has appeared during the last two years has been concerned with empirical and often heterogeneous groups of nitrogenous materials. Probably no one thing has contributed more to the advancement of knowledge in this field than the isolation of definite nitrogenous bodies from plant tissues. The work of Vickery, Pucher & Clark (57, 58) and Greenhill & Chibnall (21) is notable in this respect.

Glutamine.—The first group of workers found that the leaves and stems of tomato plants extracted with boiling water yielded more ammonia than comparable tissue which had been dried in a rapid stream of air at 80°. The substance which decomposed on boiling and yielded ammonia was glutamine. This form of amide nitrogen was determined on suspensions of dried tissue in a phosphate-borate buffer at pH 7.0 and hydrolyzed for two hours in a boiling water bath. The identity of the amide in tomato was established by direct isolation according to the procedure of Schulze and Bosshard. Using the same method of isolation, details of which are given, they readily obtained, from the roots of the common beet, yields of glutamine of the order of 80 per cent of the total amount present (58).

Ammonium and nitrate assimilation.—Vickery, Pucher & Clark ascertained further that the glutamine content of tomato leaves and stems from plants grown with ammonium sulphate in sand culture was over twice that of plants supplied with nitrate. These results are in accord with those of Greenhill & Chibnall (21) who discovered that perennial rye-grass, when heavily fertilized with ammonium sulphate, produced glutamine in such quantities that an exudate, which consisted largely of crystalline glutamine, dried on the upper parts of the leaf blades.

In apparent harmony with the foregoing it seems to be quite generally true that when plants are supplied with ammonium salts in a medium adequately aerated and at a pH value that does not drop

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much below 6.0 there is, as long as carbohydrate reserves are available, very rapid synthesis especially of amide nitrogen, but also of amino acids and other forms of soluble organic nitrogen. Comparable plants supplied with nitrate at a pH value that does not become much more alkaline than pH 6.0 are relatively much slower in the synthesis of these materials and consistently relatively low in amide. Such would seem to be indicated by the work of Clark & Shive (6) with tomato, Davidson & Shive with peach (8), and Tiedjens (55) and Nightingale (38) with apple. In the case of the fruit trees mentioned the initial synthesis of amino acids and amide nitrogen took place mainly, if not exclusively, in the fine rootlets. In tomato this occurred in both roots and tops (6, 55). Unless supplied by new synthesis there must obviously occur oxidation and decrease of carbohydrates or their derivatives in the process of amino acid or amide formation from an inorganic nitrogenous salt. Associated with more rapid synthesis of organic compounds of nitrogen from ammonium than from nitrate there occurred in tomato (55) and apple (38, 55) greater decreases in carbohydrates than in the plants of the corresponding nitrate cultures.

Burkhart (4) compared different leguminous seedlings grown in darkness in nutrient solutions with and without ammonium sulphate. Ammonium accumulated in the tissues of the plants and was associated with injury only when the concentration of sugars became low in the seedlings. These results may be compared with those of Prianschnikow (47) and Ivanova (27) who supplied etiolated seedlings with ammonium nitrate. While carbohydrates were apparently available ammonium was absorbed faster than nitrate, but, associated with extreme etiolation and accompanying carbohydrate deficiency, they reported excretion of ammonium into relatively alkaline nutrient media.

In contrast to nitrate, ammonium commonly does not accumulate in plants when carbohydrate reserves are available except in the case of very acid plant organs such as the petioles of rhubarb (49, 23). In the tomato plants referred to (6, 55) nitrate accumulated when the external source was abundant; ammonium did not, although the plants were liberally supplied with ammonium sulphate. Similar responses were observed in apple and peach except that the respective inorganic nitrogenous ions were limited mainly to the fine rootlets, the organs chiefly concerned in the initial phases of protein synthesis in these plants (38, 55).

Fife & Frampton (17), in experiments dealing in part with relationships of ammonium and amide nitrogen, placed beet plants in an atmosphere containing from 20 to 60 per cent of carbon dioxide. During a period of an hour the plant sap became less acid; associated with the decrease in acidity there was diminution in amide and a corresponding increase in ammonium nitrogen sufficient to account for the lowered acidity of the sap. Some of the plants were shifted back to usual atmospheric conditions; after about an hour the pH value of the plant sap returned to normal accompanying which there was a decrease in ammonium and a corresponding increase in amide nitrogen. Prolonged treatment with 80 per cent carbon dioxide resulted in formation of ammonium not only from amide but from other soluble nitrogenous materials. The ultimate effect of carbon dioxide was, however, that of increased acidity.

Nitrite assimilation.—Under usual conditions of nitrate nutrition, nitrite is not found in the plant at all, or only in minute quantity. Demonstrable amounts of nitrite may, however, be readily obtained in the tissues of nitrogen-deficient plants during the first flush of nitrate reduction following an application of nitrate (15).

Of considerable interest, therefore, is the work of Dikussar (13) who grew maize with nitrite as the sole external source of nitrogen, at a concentration of 200 mg. of nitrogen per liter of nutrient solution at a pH of 7.0, without injury to the plants. Lower pH values were less favorable. Eckerson (15, 16) found that extracted plant sap reduced nitrate to nitrite most vigorously at pH 7.2.

In comparison of cultures supplied with nitrate, nitrite, and ammonium, respectively, Dikussar noted the usual tendency for nitrate to accumulate in his plants whereas nitrite, like ammonium, occurred only in low concentration. In both roots and stems the highest percentage of amide nitrogen as well as total soluble organic nitrogen was found in the plants receiving ammonium. The nitrite group was only slightly lower and in striking contrast to the low values recorded for the nitrate series. A relatively high proportion of the elaborated nitrogen of the nitrate-supplied plants was present as protein (insoluble nitrogen); amino acids, rather than amide nitrogen, predominated.

Many additional papers on ammonium and nitrate nutrition have appeared and have considerable agricultural or nutritional interest but are not pertinent to this report. Murneek (37) presented a review of the literature on asparagine and related compounds and Loew (32) a discussion of theories of protein metabolism.

Factors affecting assimilation of ammonium and nitrate.—Werner, working with potato (*Solanum tuberosum*) in sand culture, shows that the synthesis of organic nitrogen from nitrate or ammonium is greatly limited by low temperature and by short photoperiods. Except that inorganic nitrogen accumulated in his plants effects of a short day or low temperature were otherwise similar to those obtained when the external supply of nitrogen was limited: the organic nitrogen was low, carbohydrates comparatively high and the dominant growth phase that of tuberization. Conversely, high temperature and long days (short nights) coupled with an abundant external supply of nitrogen, resulted in a high concentration of organic nitrogen in the plant, a low percentage of carbohydrates and little or no tuberization [cf. Eckerson (16)].

Low growing temperatures for fruit trees also have been found to retard greatly the synthesis of amide and amino acid nitrogen from nitrate and ammonium salts. Coupled with the low rate of organic nitrogen synthesis, carbohydrates accumulate owing to the decrease in the rate of their utilization in protein manufacture and to a relatively high rate of assimilation but a low plane of respiration of carbon dioxide (40).

As reported by Werner (61) and Eckerson (16) one of the dominant effects of a short photoperiod on many plants is the decrease in rate of organic nitrogen synthesis from nitrate or ammonium. The use of Mazda lamps (not necessarily of high light intensity), which supply mainly the longer wave lengths, greatly accelerates protein synthesis (16) when employed to lengthen the photoperiod, as is frequently done in commercial greenhouse culture (63). The foregoing may be compared with the preliminary report by Tottingham & Lease (56) in which the blue and longer ultraviolet radiations from a carbon arc promoted the assimilation of nitrate to a greater extent than the predominantly long waves of Mazda lamps.

Several investigations (8, 24, 41, 42) have included studies of the effects of various environmental factors on nitrate assimilation in the soy-bean plant. Rapid assimilation of nitrate was indicated by a high concentration of organic nitrogen in the plant and it was associated with a detrimental effect on nodule formation. This was particularly apparent under conditions of limited carbohydrate synthesis as brought about by shading (41) or by a short photoperiod when the latter was coupled with long, hot, summer nights (24). The depressing effect on nodule development and nitrogen fixation was considered

to be explained (18), at least in part, by the consequent lowering of the carbohydrate concentration in the plant through synthesis of organic nitrogen from nitrate. When the carbohydrate level was raised (e.g., growing the plant in an atmosphere containing additional carbon dioxide) the effects previously correlated with a high percentage of organic nitrogen were to some extent overcome. The quality of growth of the soy-bean plant (and accompanying nodule development) was apparently more intimately associated with the organic nitrogen and carbohydrate content of the plant than with any particular factor or factors of environment.

Effects of comparatively low temperatures on nitrogenous fractions are recorded in studies of hardiness or resistance to cold. Several workers (12, 20, 62) offer evidence indicating that increases in soluble organic nitrogen occur, owing to proteolysis, when plants are shifted to temperatures of about 2°. In roots of red clover (20) the principal increases occur in the amino, amide, and residual nitrogen fractions, apparently at the expense not only of protein but of the heterogeneous basic nitrogen fraction (phosphotungstic precipitate). Gassner & Franke (19) supplied wheat and rye plants with nitrate at temperatures of 20°, 10°, and 5°. An attempt was made to analyze the leaves at the same stage of development (not the same age). In contrast to the investigations cited above (12, 20, 62), it was found that protein, expressed as percentage of green weight, increased with decrease in temperature and soluble nitrogen increased slightly. A situation perhaps somewhat similar was observed in the current roots of apple and peach (39). It was apparently correlated with a high proportion of meristematic tissue (notably high in insoluble nitrogen) and a slow rate of differentiation and maturation of secondary elements.

Translocation and seasonal changes.—Loomis (33) reports that inorganic nitrogen is restricted to the roots in two-year-old poplar and fifteen-year-old apple trees. Girdling the phloem temporarily checks the movement of organic nitrogenous compounds. Similar transfer of materials from roots to tops of apple trees is prevented by a temperature of 10°, but under the same conditions there is, in the rootlets, synthesis of amino and amide nitrogen from both nitrate and ammonium salts (38).

Mason & Maskell (34), in continuation of their work on cotton, report that under conditions of nitrogen deficiency there is a translocation of nitrogen from mature leaves and base of the stem to young

vegetative organs and, with flowering and boll development, nitrogen is withdrawn from vegetative tissues.

The seasonal cycles of ten nitrogenous fractions in the wood, bark, and leaves of terminal shoots of apple trees fertilized with nitrogen and unfertilized are given by Karmarkar (30). The results, of considerable horticultural interest, are presented on a percentage and absolute amount basis as correlated with the condition of growth exhibited by the shoots at the time of each monthly analysis.

Beginning with tobacco seedlings and analyzing at intervals until maturity, Vickery *et al.* (59) present data showing the absolute amounts and percentages of organic acids, carbohydrates, and nitrogenous materials in various organs. The nitrogen determinations included both soluble and insoluble nitrogen, nitrate, nicotine, ammonium, asparagine-amide, glutamine-amide, amino, and peptide nitrogen. The relative proportions of the simpler fractions fluctuated materially, but, in general, the nitrate in the leaf diminished and that in the stem increased during the period of rapid growth. At this stage the nicotine of the leaves had increased while that of the stem remained constant. At the period of reproduction, one-third of the nitrogen of the entire plant was translocated in large part from the leaves to the seed pods. However, the quantity of nicotine in the stems remained constant while that in the leaves increased. Iljin (26) reports that nicotine, present in the immature tobacco seed, is absent at the final stage of ripening.

Herndlhofer (22), working with four-year-old coffee plants, analyzed the roots, stem, leaves, and fruit at monthly intervals. His results, expressed on a percentage basis, include determinations of protein, caffeine, amino, and amide nitrogen. Caffeine was found in all parts of the plant. As ripening progressed, there was a decrease of caffeine, amino, and amide nitrogen in the fruit with a corresponding increase in protein.

Seeds and storage organs.—Over a period of ten years Russell & Bishop (50) noted a consistent correlation between the amounts of the individual proteins and the total nitrogen concentration of barley grain as grown under various field and environmental conditions. A relatively low percentage of total nitrogen in the grain was associated with a high proportion of globulin and a low concentration of hordein nitrogen. Conversely, when total nitrogen was high in the grain, there was proportionately less globulin and a high percentage of hordein nitrogen. The proportion of glutelin was practically constant.

In wheat McCalla (35) found that the kernels from cultures with a limited nitrogen supply had a total nitrogen content of 2.9 as compared to 3.7 per cent in seeds from plants that received a liberal amount of nitrate. The kernels of the latter group were shriveled. It is suggested that this was due to a lack of carbohydrates; at least analyses of the vegetative organs indicated a high concentration of amino and amide nitrogen but a low percentage of sugars, apparently owing to rapid assimilation of nitrate. Despite the fact that the amount of total nitrogen was influenced by nutrition the proportion of prolamine to alkali-soluble protein (gluten) was the same for both lots of grain.

Wheat grain which was daily harvested from early immature stages to maturity was examined by Teller (54). As the seeds developed there was a continuous increase of gliadin and decreases in the proportions of glutenin and non-protein nitrogen. There were also slight decreases in the proportions of albumin and globulin. These changes were associated in part with development of the endosperm which contained a much larger proportion of gliadin and a smaller proportion of glutenin than the bran.

A similar study of corn kernels by Zeleny (64) showed that the concentration of globulin and glutelin remained constant throughout the growth period. Prolamine was nearly absent in the very immature seeds but accumulated rapidly as they matured. The increase in prolamine was accompanied by a proportional decrease in water-soluble non-protein nitrogen, various fractions of which were determined.

Blagoveshchenskii & Yurgenson (3) report that wheat flour contains enzymes possessing a solvent action on wheat proteins which is greatest at pH 8.5. It is interpreted as a disaggregation phenomenon rather than hydrolysis since there was no increase in amino nitrogen. Results of analysis of sunflower-seed globulin are also presented (2).

Jodidi (28) has determined the distribution of the non-protein nitrogen of the Alaska pea. He obtained pyrrolidone-carboxylic acid from the fat-free meal of these seeds by extraction with 92 per cent alcohol and subsequent oxidation of the extract. It is suggested that this acid traces its origin from glutamine.

An alkali-soluble protein material from orange pulp has been isolated by Sinclair *et al.* (51) and the nitrogen distribution determined by the Van Slyke procedure.

Stuart & Appleman (53) observed very little change in the nitro-

gen distribution of potatoes stored at 2°; at a temperature of 22°, with development of sprouts, slight proteolysis occurred. There was, however, considerable variation in different parts of the tuber, the non-protein nitrogen being higher in the medulla than in the cortex. In changes taking place in the development of a cork cambium and wound periderm the protein and basic fractions increased apparently at the expense of amino nitrogen while the amide fraction remained constant [cf. Denny (11)].

Paech (43, 44), in experiments with leaves and seeds, records that proteolysis is closely associated with respiration in the presence or absence of oxygen. With death of tissues the rate of protein decomposition is apparently accelerated if conditions, such as acid sap, do not inactivate the proteolytic enzyme presumably responsible for the reaction. He records protein synthesis in carbohydrate-deficient leaves by infiltration with glucose. He suggests that lack of either monosaccharides or ammonium may prevent new synthesis of proteinaceous materials. In a recent review Mothes (36) summarizes some of his work of the past few years which is in part pertinent to the preceding discussion.

Kiesel *et al.* (31) made extensive fractionations of protein preparations of the tubers of potato, the roots of two varieties of beet, and the seeds of two kinds of melons as well as of the leaves in each lot. The preparations, probably not individual proteins in all cases, gave significant differences in the amounts of the various amino acids determined [cf. Chibnall *et al.* (5) and Davies (10)]. Pollard & Chibnall (46) found that the proteins of several pasture grasses all contained cystine but that the protein of lucern leaf was particularly rich in this compound.

Methods.—A review of methods for the determination of relatively simple nitrogenous materials is presented by Phillips (45). It is apparent, however, that for efficiency and reliable results methods must be critically tested using the plant material concerned, although in any schedule of analysis there are some recent observations which should be considered.

In the presence of glutamine Pucher *et al.* (48) recommend determination of ammonia by distillation at 40° with a borate-sodium-hydroxide mixture used in conjunction with a phosphate buffer solution. The loss of glutamine on boiling, with consequent increase in the yield of ammonia, has been mentioned (57, 58). On the other hand, fresh tissue dried rapidly in a current of air at 80° leaves the

distribution of the simpler nitrogenous fractions unaffected (59). Further work is needed, however, to determine the total yield and quality of non-coagulable nitrogen from fresh as compared to rapidly dried tissue.

The presence of cyanogenetic nitrogen in some plants materially complicates analytical procedure. Its significance in metabolism is not apparent. Methods employed in handling such material are described in detail by Davidson & Shive (7, 9) and may be compared with determinations of this fraction by Acharya (1), and Juillet & Zitti (29). Douw & Rimington (14) report the isolation of an apparently new cyanogenetic glucoside from *A. lasiopetala*.

Stuart (52) reports that when the Van Slyke determination for alpha amino nitrogen was applied to extracts obtained by aqueous or alcoholic extraction (without prior removal of other nitrogenous fractions) the presence of the acid derivatives of the phenols from the vegetative tissues of apple gave a yield of gas that was 80 per cent too high. He recommends low temperature distillation with calcium oxide to remove the interfering tannins as well as ammonia [cf. Hulme (25)]. Stuart also found, in accord with Webster (60), that alcoholic storage of plant extracts resulted in marked increases in ammonia and decreases in alpha amino nitrogen. The decreases were not entirely accounted for by the increased yields of ammonia.

As usual, accurate physical descriptions of the plant or organs analyzed have been omitted in many cases, although such data would often facilitate the reconciliation of seemingly aberrant results. The statement that normal or average plants were employed for analysis means little; but a record, for example, of size, shape, and color of leaves, length and diameter of stem, relative succulence, and indices of vegetative or sexual reproduction, permits agriculturalists to use analytical data which otherwise would be impossible of agronomic interpretation.

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PINEAPPLE PRODUCERS' CO-OPERATIVE EXPERIMENT STATION
HONOLULU, HAWAII

THE RÔLE OF SPECIAL ELEMENTS (BORON, COPPER, ZINC, MANGANESE, ETC.) IN PLANT NUTRITION*

By P. MAZÉ

Pasteur Institute, Paris, France

The readers of the *Review* are acquainted with the principal aspects of the questions of plant nutrition through the works of Hoagland (8), Lundegårdh (11), and Steward (33). This article will describe the rôle of some special mineral elements, such as boron, copper, zinc, manganese, etc., in the higher plants.

These elements are to be found in the plant in very small quantities; they act as stimulants, or may be poisonous or indifferent in effect. The rôle of a stimulant is not far removed from that of a nutrient element if mineral nutrients only are taken into consideration; what seems to distinguish the stimulants is their ability to act in very small doses. If their concentration in the nutrient medium exceeds a certain limit, they become inhibitive, that is to say, toxic. An inhibitive effect can also be produced by a nutrient element when its concentration rises above a certain limit and becomes incompatible with physiological action. Quartaroli (23) assigns to the special elements the rôle of catalysts which act as economizers of energy by regulating physiological functions. The vitamin content of a plant seems to be in proportion to its richness in such catalysers.

The best nutrient media are those which contain all the elements essential to the plant in the proportions corresponding to those of the ascending sap. These conditions are not realized, even in the most fertile soils. Their study constitutes the most important part of plant physiology both from the theoretical and practical point of view. When one attempts to realize these conditions one observes that a complete medium must include a great number of special elements that cannot coexist in the solution in a soluble state; a complete medium often contains a certain amount of insoluble elements. The problem of their absorption must be elucidated from a particular point of view. A plant may be deprived of these special elements though in a soil which contains sufficient quantities of them. Hoagland (8) and Lundegårdh

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(11) remark that the phenomena that accompany absorption cannot be too carefully investigated.

Absorbing function of roots.—In a recent article, Mazé, Mazé, & Anxionnaz (13) have taken up this question, employing the old method of two independent solutions in which the roots of the same plant are divided into two separately immersed bundles of about equal surface area. The method gives accurate results when one takes care to avoid the intervention of microbes during the time that the experiments last (two to five days, according to the extent of sunshine). It is impossible to divide the roots into two bunches of equal surface area, but one can easily determine their relative proportions by immersing the two bundles into two test tubes filled with the same solution. When the solutions are different the relation between the surfaces of absorption does not intervene. The results depend on the composition and on the degree of concentration of the mineral medium in which the plants have been developed. The principle of this method consists in nourishing the plants with one of the root bundles bathed in the original nutrient solution, the other being immersed in a control solution containing but one soluble salt.

Maize plants grown in the mineral solution of Mazé (14) until they gave a dry weight of 2 to 5 gm. were employed. The experiments were made with four series of plants developed previously in four mineral solutions differing as follows in the nature of their nitrogen compounds:

(1) NH_4NO_3 ; (2) NaNO_3 ; (3) NH_4Cl ; (4) $(\text{NH}_4)_2\text{SO}_4$. Each of these nutrient solutions was employed in three different concentrations: S_3 , S_1 , S_4 . The different subscripts indicate the degree of concentration in comparison to the reference nutrient solution with a concentration of 1; a fourth plant had only distilled water, S_0 . The following control solutions with only one compound were employed: (a) NH_4NO_3 , 0.03 per cent; (b) NH_4Cl , 0.03 per cent; (c) $(\text{NH}_4)_2\text{SO}_4$, 0.02 per cent. In this way each of the solutions, *a*, *b*, and *c*, was given to four plants nourished with one of the solutions, 1, 2, 3, 4, employed in four degrees of concentration: S_0 , S_3 , S_1 , S_4 . The volumes of the solutions employed were 320 cc.; the quantities utilized by the plants varied from 60 to 120 cc. for each bundle of roots, both for those immersed in the control solutions, *a*, *b*, and *c*, and for those in the complete solution, S_3 , S_1 , S_4 , or S_0 .

The ratio of cations and anions being equal to 1 in the control solution at the beginning of the experiment, it was found that NH_4^+ ,

NO_3^- , Cl^- , and $\text{SO}_4^{=}$ were absorbed in unequal quantities depending upon the nutrient solution, 1, 2, 3, or 4, and upon its concentration, $\frac{1}{4}$, 1, 4, or 0. For the four plants of maize receiving solution 1 (NH_4NO_3), on the one hand, and the control solution (a), on the other hand, the ratios of NH_4^+ and NO_3^- ions absorbed were for S_0 , 1.02; for S_2 , 0.91; for S_1 , 1.08; and for S_4 , 1.22. In the group where the control solution (a) was replaced by (b), the other conditions being identical, the ratios of NH_4^+ and Cl^- ions absorbed were: for S_0 , 1.80; for S_2 , 1.12; for S_1 , 1.5; and for S_4 , 1.24. A third lot of plants for which solution (c) was employed as a control solution, all other conditions being the same, gave the following ratios for ion absorption using NH_4^+ and $\text{SO}_4^{=}$: for S_0 , 1.78; for S_2 , 1.72; for S_1 , 1.48; and for S_4 , 1.65. The ion NH_4^+ was absorbed everywhere in greater quantities than the ions NO_3^- , Cl^- , or $\text{SO}_4^{=}$, excepting in the first group for S_2 , where the ratio was 0.97. The situation was different with the plants developed in solution 2 (NaNO_3), solution 3 (NH_4Cl), or solution 4 [$(\text{NH}_4)_2\text{SO}_4$]; sometimes it was the anion that was absorbed in greater quantities, at other times it was the cation. For example, the maize developed in solution 3 (NH_4Cl) gave with the control solution (b) (0.03 per cent NH_4Cl), for S_0 , 0.5; for S_2 , 6.79; for S_1 , 1.23; and for S_4 , 0.62. The maize developed in solution 4 [$(\text{NH}_4)_2\text{SO}_4$] gave with the control solution (a) (NH_4NO_3) for S_0 , 1.05; for S_2 , 0.87; for S_1 , 0.87; and for S_4 , 1.20. In all these experiments we have to do with an absorption of ions; still we might point out that the quotient of cations to anions absorbed has proved to be equal to 1 in two cases. The possibility of realizing solutions of such concentrations as to give a quotient of 1 does not permit us to conclude that we are in the presence of a molecular absorption, nor can we extend these results to all higher plants because of the capacity of adaptation that the living cell possesses in relation to its medium. Another interesting conclusion appears from the results that we have recorded: to each of the complete nutrient solutions, 1, 2, 3, and 4, which differ only in the form of inorganic nitrogen, there correspond plants whose roots possess different powers of absorption. This power varies also according to the degree of concentration of each nutrient solution. This signifies that a definite physiological type corresponds to each nutrient medium.

Arrington & Shive (1) have also investigated the problem of anionic and cationic nitrogen in young tomato plants, ten days old. At pH 4 the absorption took place in a ratio of 0.85 to 3.95 mg. and

at pH 7 the ratio was 2.64 to 3.1 mg.; the quotient always remained below 1 during the vegetative cycle.

Experimenting upon the large multinucleate cells of *Valonia* Osterhout (20) reports that NH_3 or NH_4OH is directly absorbed, most probably by combining with some protoplasmic substance; the ammonium ion, on the contrary, is absorbed in small quantities. If we add to these factors the influence of various others, such as permeability (34), excretion by the roots, and movements of the protoplasm, one sees that the living cell can utilize different means of absorption; for this reason the characteristics of the medium in which the roots are placed must be thoroughly defined. This precaution is still more necessary when the special elements are concerned, as these are often present in an insoluble form.

Migration of mineral elements in the plant.—Vladescu (36) has studied the variations in the principal elements of the ash during the development of tobacco under the conditions of field culture. The results are extended to the whole plant (biometric method). The mineral elements as well as the organic constituents increase up to a maximum which coincides with the flowering and the maturity of the plant. From this point on the mineral matter diminishes, the quantity returning to the soil being greater than that absorbed; the total diminution was 16.4 per cent of the attained maximum. Iron, manganese, and silicon do not participate in this negative migration. Vladescu (36) made comparative determinations on cultures of tobacco in which manganese was employed in excess: 16 kg. of $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ per 1000 sq.m.; development was retarded about fifteen days as compared with that of the normal crop.

Influence of special elements upon the development of plants.—Scharrer & Schropp have made a series of important investigations (24 to 28) upon the influence of manganese, iron, zinc, cadmium, molybdenum, tungsten, chromium, and vanadium on the germination of cereals, corn, and peas. The germination and culture took place, partly in 800 gm. of purified sand to which a suitable quantity of Richter's solution (320 to 350 c.c.) was added, and partly in a liquid medium (Richter's solution) in 2-litre flasks. Control plants without the elements under investigation were cultivated under the same conditions.

Manganese (24) was added to 800 gm. of sand at variable doses, up to 10 mg., in the form of $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$. The cultures of wheat were stimulated by the manganese; the effect displayed two maxima,

one at a dosage of 10^{-10} , the other at 10^{-1} m.eq. of manganese; at a level of 10 mg. the manganese was visibly toxic. The effects were about the same on barley and rye. The maize was cultivated in Richter's medium without sand to study the interrelations of iron and manganese. When iron was replaced by manganese the maize did not develop as well as when the two metals were absent; the best results were obtained when iron and manganese were utilized in the proportion of 7 to 1.

Zinc and cadmium were employed in the form of $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ and $3 \text{CdSO}_4 \cdot 8 \text{H}_2\text{O}$ (25). Zinc was a stimulant for the plants under experimentation with the exception of oats. The maximum effect was obtained for maize with 10^{-8} m.eq. of zinc per litre of Richter's solution and with 10^{-7} for wheat and peas, and 10^{-6} for rye and barley. Oat plants that succeeded best, in comparison with the control plants, were grown in the presence of 10^{-5} m.eq. per litre. In plants in which zinc had a favorable influence the yield diminished, beginning from optimal doses, until when 1 m.eq. per litre was reached, the zinc was clearly poisonous. Employed in equivalent doses, cadmium was a weaker stimulant than zinc; it was also more toxic.

The effects of molybdenum and tungsten (26), administered as $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$ and $\text{Na}_2\text{WO}_4 \cdot 2 \text{H}_2\text{O}$, were investigated upon maize plants which were transferred after three days' germination into 2-litre bottles with Richter's solution in the presence of molybdenum or tungsten in doses from 10^{-10} m.eq. to 100 mg. Molybdenum always proved to be toxic; tungsten was a stimulant up to a dosage of 1 mg.

Chromium (27) was employed in the form of $\text{Cr}_2(\text{SO}_4)_3$ in doses varying from 10^{-10} m.eq. to 100 mg. per 800 gm. of purified sand, to which pure water had been added. The cereals, maize, and peas developed in these media, were not, in general, sensibly affected after ten to twenty days of germination except when in the presence of higher doses of chromium. Small doses had a slightly stimulating action with maximum of effect at 10^{-4} mg. of chromium. However, among the cereals, the development of wheat was delayed in the presence of 100 mg. of chromium; that of peas was almost inhibited; maize cultivated for two months in 2 litres of Richter's solution, in the presence of the same doses of chromium, was inhibited by doses greater than 10 mg. The growth, as noted at various intervals during one month, showed a slight stimulation in doses up to 1 mg., a faint toxicity at 10 mg., and inhibition at 100 mg.

As $\text{Na}_2\text{CrO}_4 \cdot 10 \text{ H}_2\text{O}$ chromium was still more poisonous in a liquid medium, but in sand it acted as a stimulant at 0.1 to 1 mg. Chromium would not replace iron; the authors nevertheless observed a slight stimulation by feeble doses of chromium in a solution deprived of iron; but due consideration must be given to the fact that the seed contained a small amount of iron.

The effect of vanadium on the development of plants is the subject of the final article by Scharrer & Schropp (28). Placed in 800 gm. of sand, to which pure water containing 10^{-10} to 100 mg. of vanadium as $\text{NaVO}_3 \cdot 4 \text{ H}_2\text{O}$ was added, the seeds developed normally in the presence of doses from 1 to 10 mg. of vanadium. Wheat was again the most resistant; it was not influenced by 100 mg. of vanadium. But the seeds of peas gave no germination in the presence of this quantity. From the yields obtained afterward the detrimental influence of vanadium was evident in doses of about 1 mg.; nevertheless a slight stimulation was observed in the presence of weak doses, 10^{-4} and 10^{-2} mg. Cultivated in water in the presence of increasing doses of vanadium, maize regularly gave decreasing yields.

The influence of manganese and iron upon the development of flax and their rôle in the production of chlorosis in that plant is the subject of very interesting experiments by Scholz (29). The flax was cultivated in vessels containing 4 kg. of purified glass sand to which were added KNO_3 , 0.5 gm.; KH_2PO_4 , 0.3 gm.; K_2SO_4 , 0.2 gm.; and NaCl , 0.2 gm. Half of the vessels received also 10 gm. of calcium carbonate. Half of each of these two groups received 0.1 gm. of $\text{MnCl}_2 \cdot 4 \text{ H}_2\text{O}$, while the remainder received $\text{Fe}_2(\text{SO}_4)_3$, 0.1 gm., or $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$, 0.2 gm. In the presence of iron and calcium carbonate the vegetation was normal and the crop developed in thirty days was healthy. In the absence of iron the plants were very chlorotic after fifteen days with calcium carbonate alone; those that developed in the presence of iron alone were not chlorotic, but the yield was lower than in the presence of iron and calcium carbonate. Imminent chlorosis was avoided by the addition of iron to the nutrient solution; an evident chlorosis could be cured in the same manner.

The manganese employed in the dose indicated above was toxic; it was slightly stimulating in very small quantities, but the results do not indicate whether it is indispensable to the development of flax. The observations on the action of the magnesium have not been published.

Loomis & Wilson (12) have determined the action of boron and

of iron on tomato plants. It is known that tomatoes do not develop in mineral solutions deprived of boron. The optimal quantities of boron in the form of boric acid vary, according to Loomis & Wilson, from 0.1 p.p.m. to 0.5 p.p.m., but the results are not regular. There exists no correlation between the effect of boron and that of iron in the presence of 40 p.p.m. to 160 p.p.m. of ferric chloride and 0.1 p.p.m. to 0.5 p.p.m. of boron as boric acid; all the plants are destroyed. The pH of the toxic solutions was 3.5 and it was not affected by the quantities of boron employed.

Nag (19) attributes the chlorosis of pine trees (*Abies balsami*) to a deficiency of manganese. Van Schreven (31) describes the external and internal symptoms of lesions, induced by the absence of boron, in tobacco plants cultivated in sand or in nutrient solutions; the roots lose color, the buds are destroyed, and the leaves become chlorotic and curl, with a deposition of crystals of oxalate; carbohydrates accumulate abundantly because of a poor circulation of the sap.

Weber (37) has studied the lesions observed in orchard trees of citrus fruits, prunes, and apricots irrigated by water containing an excess of boron. The lesions are particularly important in places where the boron accumulates in the stems and leaves; their character varies with the species. The toxic action stimulates cell growth with an abnormal increase in volume, leading to a progressive degeneration of the tissues.

Boy (4) allowed seeds of rice to germinate in water in the presence of increasing toxic doses of manganese and zinc, 1, 2, 4, or 6 gm. of manganese or zinc per litre (added as the sulfate), and studied the energy yield and formation of vegetable matter in terms of the weight lost by the seeds. Germination was delayed and energy formation decreased with increase of added manganese and zinc. The formation of vegetable matter was less affected than the energy transformation.

Zlatarof (38), having studied the stimulating action of manganese and magnesium on the germination of chick peas and arachis, reports that the energetic as well as the vegetable-matter yields are not affected by manganese employed in stimulating doses. A 0.04 per cent solution of $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ was more stimulating than a 0.06 per cent solution of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, both salts being allowed to act on the seeds during five hours before their immersion in distilled water for germination. It seems that manganese acts as a coferment in the digestion of the cotyledon reserves.

Now if we examine the conclusions that proceed from all the articles reviewed, we can state that the special elements act as stimulants on some species and are poisonous for others. The distinction is quite common from one family of plants to another and even from one species to another. It follows that the quantities of special mineral elements needed are very different among various higher plants. This fact is not at all surprising but its practical consequences may be important; the problem of the fertility or the specific sterility of the soil thus presents itself in quite an interesting light (2).

Indifferent and toxic elements.—Vegetable ash contains, together with indispensable mineral elements, some others absorbable from the soil that are indifferent or even toxic to the plant. Thus, the ash reflects in a certain degree the composition of the soil. The study of the ash constituents illuminates one aspect of the problem of the comparative fertility of arable soil in relation to its content of toxic elements.

As was proved by Geilmann & Brünner (6) germanium is absorbed by plants when it exists in a soluble state in soil. In small doses it has no effect on the development of plants but becomes rapidly toxic in higher doses.

Molybdenum exists in the leaves of arborescent plants when contained in the soil. Meulen & Ravensway (18) have found it in eleven species out of twelve which they investigated. It has been estimated in the ash of 1000 leaves taken as far as possible from the same branches at different vegetative phases. The quantity of molybdenum in 1000 leaves varied from 0.0009 mg. in the green beech to 0.89 mg. in the horse chestnut. It seems that molybdenum does not migrate into the stems before the fall of the leaves.

Borzini (3) has studied the action of thallium as $TiSO_4$ on the germination of various seeds: wheat, oats, beans, peas, white mustard, lucerne, clover, buckwheat, lupine. Thallium is absorbed by the roots, delays germination, inhibits growth, and induces chlorosis. It was poisonous in all the concentrations employed.

Diseases of plants in relation to mineral nutrition.—A plant deprived of an indispensable element loses a part of its photosynthetic power even when the element in question is utilized only in infinitesimal doses; there is a diminution or total disappearance of chlorophyll; this more or less intense chlorosis constitutes the most frequent symptom of disease due to mineral deficiency. Cryptogamic diseases manifest themselves sometimes by the same symptoms, because the

cryptogams can turn aside for their own use the mineral elements indispensable to the plant; it is therefore possible sometimes to arrest the development of the fungus by furnishing the plant with the element of which it is being deprived. The attacks of insects, such as *Phylloxera*, localized in the roots, thwart the function of absorption and produce indirectly a mineral deficiency also characterized by chlorosis. The intoxication produced by the presence of toxic mineral elements in the soil finally produces the same result. If we possessed practical means of distinguishing these different causes of organic diseases and recognizing the mineral deficiencies produced by cryptogamic diseases or by the attacks of insects, we might devise efficient methods for their treatment.

The means of diagnosis exist (15); we need only place on the surface of the chlorotic leaves some drops of a very dilute solution (about 10 mg. per 100 cc.) of a soluble compound of the missing element to see, after twelve to twenty-four hours' exposure to sunshine, green spots appear in the place of the drops. When the result is negative with a given element, we must have recourse to a second or a third. It may happen that the chlorosis is due to the simultaneous deficiency of several elements; iron, manganese, and zinc can be simultaneously lacking from a soil rich in chalk or, separately, from soils poor in chalk. The experiment is not always successful when leaves, too seriously affected, are being experimented upon; the less injured must be chosen.

The experiment is still less successful when the intoxication is due to an excess of special elements, or to the presence of essentially toxic elements in the soil. Nevertheless, there exists a possibility of diagnosing an intoxication due to one of these factors (16). The method is to utilize the crude juice, extracted by grinding normal leaves of the same species under investigation, and adding drops of this juice to affected leaves; after some hours of exposure to sunshine chlorophyll appears. A direct application of these methods was made by Mazé & Eveno (17) to the diagnosis and treatment of a chlorosis produced by a deficiency of iron in the sewage-disposal grounds of the city of Paris and which, with the exception of the grasses, extended to all plants in these areas. The application of these methods of investigation has a promising future.

The works of Chandler, Hoagland & Hibbard (5) of the University of California inaugurated a series of researches on utilization of impure iron sulfate and zinc in the treatment of such citrus

diseases as "mottle-leaf," or "little-leaf" (in the case of deciduous trees).

The history of these researches has been set forth by Parker (21); other results acquired in various research institutes on a practical treatment for mottle-leaf are also described. The process of spraying the leaves with a mixture of zinc sulfate, hydrated lime, and water, having been efficient in the majority of cases, the program for the research work of 1934 was planned to elucidate the following points:

- a) The effects of environmental conditions, fertilization, and other cultural practices and rootstock relations upon the occurrence of mottle-leaf and on the effectiveness of zinc treatment.
- b) The most satisfactory spray formula and dust material for treatment, and the most desirable time of application.
- c) The results obtained by the introduction of solid particles of zinc into the trunks of trees.
- d) The effectiveness of relatively insoluble zinc compounds when applied to soil.
- e) The duration of effectiveness of treatment by means of various methods.
- f) The effect of repeated treatment.
- g) The relations of zinc treatment to the best control program.

The basic formula for the mixture (zinc sulfate, hydrated lime, water), confirmed by the earlier tests, was the following: Formula 10-5-100; 10 pounds of $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 5 pounds of hydrated lime, 100 gallons of water. The results acquired during the investigations of 1934 are as follows [Parker (22)]:

Solutions containing 5 pounds of zinc sulfate in 100 gallons of water without lime produced serious injuries to the succulent parts of the trees. In a concentration of 1 pound per 100 gallons the young oranges on the coast were damaged more severely than those in the interior of the country.

The precipitation of zinc was thus recognized to be necessary. The constant usage of the formula 10-5-100 gave decisive results; smaller proportions of lime were less successful; higher proportions were not more efficacious. Even when the concentration of zinc is lower the proportion of 10/5 with respect to lime must be maintained. Light sprayings were efficacious. Rain did not wash away the active substance prematurely when the precaution was taken to incorporate into the mixture a little blood protein. The time of treatment did not sensibly influence its efficacy, but experience indicates that the earlier treatments produce the most striking effects and at the same time

improve the quality of the crops. It was not proved that further improvement could not be achieved.

Isaac (9) describes the chlorosis of young peach trees in the virgin soils of South Africa. In comparative experiments Isaac treated the affected trees with sulfates of copper, potassium, and manganese incorporated into the soil. Of the three compounds it was copper sulfate that gave the best results; potassium sulfate produced a significant amelioration, but manganese sulfate was without effect. The copper sulfate was used in quantities of 4 kg. per 1000 sq.m. at a depth of 0.1 m. These observations do not indicate the exact nature of the mineral deficiency. An application of the process of diagnosis indicated above, and recourse to spraying of the leaves with appropriate solutions would have probably given more accurate results. The same conclusion can be drawn from numerous articles devoted to the diseases of the sugar beet: disease of the root heart attributed to boron deficiency; chlorosis and mosaic attributed to viruses, to manganese deficiency, and to fungi, etc. Spraying of the leaves with specific solutions may be efficacious even if the disease is due to a microbic infection. It happens often that cryptogams invade plants weakened by mineral deficiencies and thus aggravate their condition. Alimentionation of the plant by way of the leaves may cure it rapidly.

As an example of chlorosis of cultivated plants due to an excess of lime, Scholz (29) describes the chlorosis of the blue lupine and of the serradelle. Chlorosis is always more severe at the stage of germination and can be corrected by iron. Serradelle is more sensitive to the excess of lime than blue lupine. The latter has a great need for manganese which it easily absorbs from soils containing soluble iron. In the presence of an excess of lime, manganese aggravates the chlorosis resulting from iron deficiency. The seed of the blue lupine contains a sufficient amount of manganese for its development but the plant becomes chlorotic if not provided with manganese; this chlorosis is different from the one produced by an excess of lime.

Resistance of the plant to cryptogamous infections in relation to mineral nutrition.—We may conclude from the facts mentioned in review that the mineral elements play a necessary rôle in maintenance of the plant. It is generally the special elements that are of such significance, in contrast to the mineral nutrients that the plant utilizes in large quantities, such as potassium, phosphorus, magnesium, and sulfur. But in reality the lack of any one of the latter makes all development virtually impossible, while a culture can flourish in the

presence of very small quantities of the special elements if the pH of the soil does not render their absorption impossible or if cryptogamic infections do not deprive the plant of them. In certain cases we can succeed in checking a cryptogamic disease by causing the leaves to absorb the deficient element. The special mineral elements thus become important to the plant as factors in immunity (16). They can intervene, thanks to their antiseptic properties, as do the elements employed therapeutically and for the prevention of certain diseases in man and animals.

Sempio (32) has shown that a solution of cobalt nitrate [$\text{Co}(\text{NO}_3)_2$], in a concentration of M/15,000, hinders the formation of tumors in plants of *Ricinus*, inoculated with *Phytomonas tumefaciens*, without injuring the plant itself. In consequence cobalt augments the resistance of *Ricinus* to infection by *Phytomonas tumefaciens*. Mercuric nitrate in a concentration of M/50,000 kills this parasite but it favors the development of the tumor; it diminishes the resistance of the plant in favor of the parasite. Copper, zinc, lead, strontium, and barium act like mercury, but with less intensity. Thus we see that chemotherapy of plants requires a most careful choice of appropriate elements.

According to Germar (7), silicon, in the form of SiO_2 may also exercise a protective influence against some cryptogamic infections of the grasses. Colloidal silica, introduced into sand treated with fertilizing elements, is absorbed; it becomes deposited mostly on the membranes of the epidermal cells and the parenchyma and forms a protective shell which makes the plant more resistant to cryptogamic parasites. See also "The Rôle of Silicon in Plant Nutrition" [Streinivarsan (35)].

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PASTEUR INSTITUTE
PARIS, FRANCE

BACTERIAL METABOLISM*

By A. J. KLUYVER

*The Microbiological Laboratory
Technical University, Delft, The Netherlands*

INTRODUCTION

A review of the annual progress in our knowledge of bacterial metabolism is foredoomed to bear a fragmentary character. The unity in the biochemistry of all living organisms is nowadays so evident that advances made in special chapters of this science will inevitably also influence our insight into the biochemical behaviour of other groups of organisms.

Besides being fragmentary the survey given will also be of a most heterogeneous nature. This is a direct consequence of the fact that the bacterial kingdom is characterized by the exceptionally large diversity of the chemical abilities of its representatives, so that there is practically no problem of physiological chemistry which cannot be studied by using bacteria as test objects.

THE METABOLISM OF THE CHEMO-AUTOTROPHIC BACTERIA

The nitrifying bacteria.—An extensive contribution to our knowledge of the nitrifying bacteria has been published by Kingma Boltjes (1). Partly this paper is devoted to the refutation of previous statements regarding the rôle of heterotrophic bacteria in nitrification, and to a critical examination, from a standpoint of classification, of the different autotrophic species active in this process. Definite proof was furnished for the indispensability of calcium ions for optimal growth of *Nitrosomonas* species. Of much importance seem the results obtained with regard to the behaviour of *Nitrosomonas* and *Nitrobacter* species towards various organic substances. Although a remarkably favourable effect on the development of these organisms by the addition of *Nährstoff-Heyden* was found, yet careful experiments showed that also under these conditions the metabolism remains autotrophic, both the ordinary inorganic oxidation substrates and carbon dioxide still being indispensable for proliferation.

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With a view to this more or less paradoxical result the possibility of the occurrence of a chemomixotrophic metabolism is pointed out. The inhibitory effect of various other substances on the growth of the nitrifying bacteria proved to run parallel to their inhibitory effect on the respiration of the organisms. According to an earlier investigation of Meyerhof, glucose, which does not inhibit respiration, is an exception to this rule; the remarkable result that, indeed, under certain conditions nitrification proceeds vigorously in media containing up to 4 per cent of glucose may therefore be especially cited.

Corbet (2) studied the chemistry of the oxidation of ammonia to nitrous acid in crude cultures. Although the occasional presence of hydroxylamine and a rather regular occurrence of hyponitrous acid under such conditions is interesting, yet the interpretation that these substances would be intermediate products in the nitrification process is open to doubt as a result of the probable simultaneous occurrence of denitrifying bacteria in these media.

The colourless sulfur bacteria.—Valuable contributions to our knowledge of the metabolism of the different species of the genus *Thiobacillus* have been made by Starkey (3, 4, 5, 6). From the extensive studies of this author it results that besides two strictly autotrophic species (*Th. thioparus* Beijerinck, and *Th. thiooxidans* Waksman and Joffe) this genus contains several other species which also oxidize thiosulfate. Amongst the latter there is at least one species, *Th. novellus* Starkey, which is a facultative autotroph, while several other strains, including *Th. Trautweinii* Bergey *et al.*, are strictly heterotrophic. There proved to be marked differences in the ways in which thiosulfate is oxidized by the representatives of the various groups. In the case of *Th. thioparus* sulfate, sulfuric acid and elementary sulfur are formed; accordingly the pH of the medium decreases during incubation. The same holds for *Th. thiooxidans* except that no sulfur is formed. In contrast herewith the first products of thiosulfate oxidation by the strictly heterotrophic strains are tetrathionate and free alkali, secondary chemical reactions resulting in an accumulation of some tri- and pentathionates, sulfate, and elementary sulfur. As a result the pH of the medium at first increases; the secondary reactions subsequently cause a drop in pH. Additional proof is given that the latter type of metabolism is also met with in very common species like *Ps. fluorescens*, *Ps. aeruginosa*, and *Achromobacter Hartlebii*. These observations afford important

proof for an intimate natural relation between the truly autotrophic *Thiobacillus* species and the ordinary polarly flagellated heterotrophs.

Czurda (7) reports the isolation of two new thermophilic *Thiobacillus* species from the water and the mud of a hot spring in Pistyan (Czechoslovakia); these bacteria, which oxidize thiosulfate and other sulfur compounds, were accompanied by a new *Thiospirillum* (?) species which presumably forms hydrogen sulfide either from sulfur or from sulfate.

Iron bacteria.—A discussion of the behaviour of iron bacteria towards manganese in solution is found in a paper by Beger (8) who also describes a new iron bacterium, *Leptothrix echinata*, which seems to have a preference for oxidation of manganese. The appearance of the second part of the monograph of Dorff (9) on the iron bacteria can only be announced here.

THE METABOLISM OF THE PHOTO-AUTOTROPHIC BACTERIA

The study of bacterial photosynthesis as occurring in the purple bacteria has been continued by Roelofsen (10, 11), Gaffron (12, 13), van Niel (14), and van Niel & Smith (15).

Gaffron (12) reports in detail on the photochemical carbon dioxide reduction with fatty acids by a *Rhodovibrio* species. In these experiments conclusive evidence is given that the amount of carbon dioxide assimilated per molecule of fatty acid increases with the length of the carbon chain. As is pointed out by van Niel (14) recalculation of the data in question indicates that per CH_2 group an approximately constant quantity (0.4 mol) of carbon dioxide is assimilated, a result which is in good agreement with earlier determinations by Gaffron. The observation of Roelofsen that *Thiorhodaceae* are able to reduce carbon dioxide with the aid of gaseous hydrogen in the light led Gaffron to a closer study of the behaviour of his *Rhodovibrio* towards hydrogen during illumination. The interesting result of these experiments was that not only carbon dioxide, but also nitrate and various organic substances, viz., fatty acids and derivatives, were reduced with the uptake of hydrogen gas.

In Roelofsen's investigations special attention has been given to the metabolism of the purple sulfur bacteria in the dark. It was shown that under such conditions an "autofermentation" occurs in which unknown reserve substances of the bacteria are converted into carbon dioxide with simultaneous production of acid. Arguments are given in favour of the view that this process occurs also in the light, al-

though here its effect is veiled by the carbon dioxide assimilation. The unexpected observation was made that the nature of this autofermentation process is influenced by the previous history of the organisms; Thiorhodaceae grown in peptone media produce gaseous hydrogen in addition to the fermentation products already mentioned.

A counterpart of the dark process of autofermentation is the process which Roelofsen has named "auto-assimilation." In this process carbon dioxide is assimilated in the light with the aid of unknown products of the dark metabolism as hydrogen donors.

Furthermore, Roelofsen carried out some experiments in connection with Gaffron's claims that—contrary to the opinion of van Niel and of Muller—organic substances cannot act directly as hydrogen donors in the photochemical reduction of carbon dioxide by Thiorhodaceae.¹ Since Roelofsen did not find any indications for the occurrence of sulfate reduction in the dark, Gaffron's theory is rejected.

More recently Gaffron (13) has returned to this problem. The extensive experimental material collected is again interpreted as evidence in favour of his concept. In the light of these experiments Roelofsen's criticism can no longer be considered conclusive; yet Gaffron's results still leave room for the possibility of a different explanation of the phenomena described. It should be particularly mentioned that Gaffron is of the opinion also that the photochemical assimilation of carbon dioxide with molecular hydrogen proceeds via the formation of hydrogen sulfide. Gaffron also studied the influence of carbon monoxide and hydrocyanic acid on the metabolism.

Mention may still be made of the general survey of bacterial photosynthesis by van Niel (14). The same author, in collaboration with Smith (15), has isolated a red pigment from the purple bacterium *Spirillum rubrum* which was proved to be a highly unsaturated carotenoid of the formula $C_{48}H_{66}O_3$.

The study of bacteriochlorophyll has been continued by Fischer & Hasenkamp (16, 17) and its close relationship with chlorophyll, as already demonstrated by Schneider, has received additional support.

As is pointed out by Roelofsen the determination of the quantum efficiency of purple-bacteria photosynthesis is of considerable

¹ *Ann. Rev. Biochem.*, 4, 610 (1935).

theoretical importance. In the photochemical reduction of one molecule of carbon dioxide with the aid of hydrogen sulfide one quantum of most wave lengths will suffice to answer the thermodynamic requirements of the reaction, whereas in ordinary photosynthesis of the green plants at least 2 or 3 quanta, depending on the wave length, are required. On the other hand various theories regarding the internal mechanism of photosynthesis imply that in both cases 4 quanta are involved, a number which is in most satisfactory agreement with that found by Warburg & Negelein in the case of the green alga *Chlorella*. It is therefore clear that the experimental determination of the quantum number, whether 1 or 4, may have decisive influence on the evaluation of the various theories proposed for this process. It must be added, however, that Roelofsen's own experiments are of too preliminary a nature to settle this question.

THE METABOLISM OF THE AËROBIC HETEROTROPHIC BACTERIA

General studies on bacterial respiration.—Frey (18) has published another review of the respiratory mechanisms encountered in various species of bacteria.² The studies of Fujita & Kodama (19, 20, 21) on respiration and fermentation in various pathogenic bacteria have been continued. The influence of various factors on respiration and fermentation of *B. diphtheriae* has been investigated. From the results obtained special mention should be made of the fact that mono-iodoacetic acid inhibits respiration and fermentation to the same extent. Also changes in the pH of the medium did not lead to a complete separation of the two processes. In a second paper special attention has been given to the occurrence of cytochrome in various pathogenic bacteria. It is of importance that all strictly anaërobic species and also the *Streptococcus* and *Pneumococcus* species investigated are devoid of cytochrome; their respiration is practically not inhibited by carbon monoxide or hydrogen cyanide. The third publication brings a detailed study of the respiration of *Pneumococcus*. On the whole the results are in good agreement with those of Sevag.³ Potassium, calcium, and especially magnesium ions raise the respiration intensity. About 80 per cent of the oxygen consumed is recovered as hydrogen peroxide; the respiratory quotient was always found to be higher than 0.5, indicating that the oxidation of the substrate does not lead completely to the final products: carbon diox-

² Cf. *Ann. Rev. Biochem.*, 4, 595 (1935).

³ Cf. *Ann. Rev. Biochem.*, 4, 598 (1935).

ide and water. The degree of hydrogen peroxide formation and virulence appear to run parallel.

The study of Farrell (22) on the respiratory mechanism of *Streptococcus* species fits in rather well with the results obtained by Sevag and by Fujita & Kodama. A thermostable peroxidase was found to be present; its function, however, is normally masked by the thermolabile dehydrase mechanism in the cell. Cytochrome and Warburg's respiratory ferment were shown to be absent.

An extensive investigation on the occurrence of "indophenolase" ("Nadi"-oxidase) amongst bacteria of different groups has been carried out by Yamagutchi (23). It appears from this study that in all indophenolase-containing bacteria cytochrome-*c* was also present, whereas with a rare exception the bacteria which are free from this compound do not contain the enzyme in question. Although these results seem to indicate an intimate relationship between these two agents, yet it appears from the study of the action of carbon monoxide and hydrogen cyanide on both "Nadi"-oxidation and respiration that the two processes behave quite independently. It is concluded that, in agreement with the theory of Shibata and Tamiya, the influence of carbon monoxide on respiration is not due to the inhibition of indophenolase but to the prevention of the oxygenation of the cytochrome.

The study of the respiration of various *Rhizobium* species has been continued by Walker and collaborators (24, 25). It was found that the respiration of *Rh. meliloti* and that of *Rh. japonicum* responded almost identically to changes in the reaction of the medium, indicating a close relationship in their respiratory mechanisms. In a second communication observations regarding the suitability of various sugars and sugar alcohols as respiration substrates for each of these two species are reported; marked differences were observed. It has, however, to be taken into consideration that in these experiments the combined effect of the substrate on growth and respiration of the bacteria was determined.

Allison, Hoover & Burk (26, 27) published a preliminary report on an interesting growth and respiration factor for certain *Rhizobia*. The most important point is undoubtedly that this factor of the bios type, to which the name coenzyme-R is given, brings about an almost immediate increase in respiration intensity (100 to 400 per cent within one to two hours), whereas the growth response does not occur until several hours later.

Coenzyme-R was found to be present in practically all natural materials examined. Attempts at isolation of the principle led to a highly active preparation, although no crystalline product has yet been obtained. In the beginning it was suspected that coenzyme-R would be identical with Williams' pantothenic acid; further research has shown, however, that this is not true, nor could the factor in question be identified with any similar agent described until now. In this connection it may be mentioned that according to McBurney, Bollen & Williams (28) pantothenic acid, produced in cultures of *Rhizobium meliloti*, has a favourable effect on the development of "sterile" seedlings of alfalfa.

The respiratory metabolism of acetic acid bacteria.—As is known the respiratory metabolism of acetic acid bacteria is characterized by the incompleteness of the oxidations brought about by these organisms. A practical application of this fact for the preparation of various organic compounds was propagated thirty-five years ago by Bertrand in his classical studies on the sorbose bacterium. The increased interest in the commercial manufacture of sorbose for the synthesis of vitamin C has led to various publications in which the biochemical production of the said sugar out of sorbitol is described. Schlubach & Vorwerk (29) and also Maurer & Schiedt (30) give detailed directions for the successful preparation of sorbose with *Bacterium xylinum* (sorbose bacterium). Bernhauer & Görlich (31) made a comparative study of the suitability of *B. xylinum*, *B. xylinoides*, and *B. gluconicum* for the same purpose in continuation of some preliminary observations made by Hermann & Neuschul. The last named species led to the highest yields (over 70 per cent of the theoretical value).

Ten years ago Kluyver & de Leeuw fixed attention on the great advantages of a new acetic acid bacterium for this and similar preparatory purposes. In contrast to what holds for *A. xylinum*, cultures of this organism (*Acetobacter suboxydans*) can be artificially aerated without any danger for a further oxidation of the primary conversion products. Working with this bacterium and applying the principle outlined Böeseken & Leefers (32) report very high yields of sorbose obtained in experiments which lasted only a few days.

Since Bertrand's pioneer work *A. xylinum* has frequently been used for the preparation of dihydroxyacetone from glycerol. Virtanen & Nordlund (33) as well as Neuberg & Hofmann (34) have recently confirmed the suitability of *A. suboxydans* for this purpose.

A method for continuous preparation of gluconic acid with the aid of *Bacterium gluconicum* has been elaborated by Hermann & Neuschul (35).

A detailed study of the formation of acetic acid out of ethyl alcohol by *Bact. ascendens* has been made by Janke & Kropacsy (36). When compared with earlier investigations this work has the great merit that it is not restricted to the determination of oxygen consumption. The special reaction vessel devised (37) permitted the simultaneous estimation of ethyl alcohol and its oxidation products, for which purpose special analytical methods have been developed (38). The results obtained show decisively that under normal conditions, that is in acid media, the oxidation of ethyl alcohol proceeds in two consecutive steps: the dehydrogenation of the alcohol to acetaldehyde and the dehydrogenation of the latter to acetic acid. The theory of Neuberg which assumed that acetaldehyde would undergo a dismutation must now definitely be abandoned, because this process only occurs with appreciable velocity in a region above pH 8.4. The conditions which favour the accumulation of acetaldehyde as an intermediate product have also been determined; they comprise an acid environment and the presence of a high percentage of viable cells.

Of considerable theoretical and possibly also of practical interest are the results of the studies of Bernhauer & Irrgang (39) and of Bernhauer & Görlich (40) on the products of the oxidation of glucose by *Bacterium gluconicum*. Whilst the formation of 5-ketogluconic acid had been reported by several investigators in the experiments in question the formation of an additional reducing acid could be demonstrated. Four years ago Takahashi & Asai had obtained an analogous result with their *Bact. industrium* var. *Hoshigaki* and had expressed the opinion that this second acid would be *l*-glucuronic acid. Bernhauer & Irrgang rightly criticize this conclusion and make it probable that this acid is aldehydo-gluconic acid (*l*-guluronic acid), although a definite statement cannot yet be made.

On repeating these experiments Bernhauer & Görlich found that their bacterium had markedly changed its biochemical behaviour. The production of 5-ketogluconic acid had strongly increased and besides the aldehydo-gluconic acid apparently a third acid had been formed. This last acid could be identified as 2-ketogluconic acid, a result which is worthy of attention for several reasons. In the first place the formation of this acid conflicts with Bertrand's well-known

rule concerning the necessity of an hydroxyl group in the *cis* position for the biochemical formation of a keto group out of a secondary carbinol group. Moreover, since the 2-ketogluconic acid on decarboxylation yields arabinose, the said acid may be the natural precursor of this pentose.

That various acetic acid bacteria are also able to produce protocatechinic acid out of quinic acid is proved in another paper by Bernhauer & Görlich (41). Tanaka (42) has made a study of the behaviour of *Bacterium aceti* towards fifteen different organic acids. Only acetic, succinic, fumaric, pyruvic, and lactic acid were oxidized with appreciable velocity; in all cases the respiratory quotients observed were in agreement with the supposition of a complete oxidation to carbon dioxide and water. Neither quinone nor methylene blue was able to act as a substitute for oxygen in these oxidations.

The respiratory activities of bacteria of the colon group.—Several publications deal with the formation of indole by *B. coli*. Woods (43, 44) studied this formation from *l*-tryptophane by thick washed suspensions of the said organisms. Under conditions of continued aëration there was a quantitative conversion into indole. The oxygen uptake answered the demands of the equation: $C_8H_9N \cdot CH_2 \cdot CHNH_2 \cdot COOH + 5O \rightarrow C_8H_7N + 3CO_2 + NH_3 + H_2O$. The rate of disappearance of tryptophane was identical with the rate of formation of indole; *d*-tryptophane is broken down, if at all, only at a very slow rate. Under anaërobic conditions β -indole propionic acid was formed. No formation of indole could be observed from β -indole aldehyde, β -indole carboxylic acid, β -indole acetic acid, β -indole propionic acid, and β -indole acrylic acid. Happold & Hoyle (45) have demonstrated that the conversion of tryptophane into indole can be carried through with killed preparations of *B. coli*. They introduce the name tryptophanase for the catalytic system active in these preparations and prove its activity over a wide range of acidity (pH between 5.0 and 10; optimum, 8.5). Bacteria grown on media with high tryptophane content yield preparations with much increased potency.

Bernheim, Bernheim & Webster (46) have performed similar experiments with "resting" *B. proteus*. Suspensions of this bacterium oxidize leucine, phenylalanine, and methionine rapidly, serine, alanine, and proline rather well, but tyrosine and tryptophane more slowly. Deamination, as a rule, accompanies the oxidation. Only alanine, serine, proline, valine, and tryptophane are decarboxylated.

Nitrogen fixation by aerobic bacteria.—A sensational achievement has been reported by Bach, Jermoljewa & Stepanjan (47) who succeeded in obtaining a very marked nitrogen fixation with the aid of cell-free preparations from *Azotobacter chroococcum*. A press juice of the bacteria after filtration through a Chamberland filter and even in a dilution of 1:10 was able to fix considerable amounts of nitrogen, when mannitol or glucose and a phosphate buffer were added. The greater part of the nitrogen fixed was present as ammonia; pasteurisation of the juice scarcely diminished its fixative ability. These results are the more noteworthy since it would be the first instance of an appreciable conversion of glucose by enzyme preparations from aerobic bacteria. Confirmation of these experiments is therefore badly wanted.

Endres (48) claims to have demonstrated the occurrence of small quantities of hydroxylamine in cultures of *Azotobacter*, especially in media with lactate. His conclusion that hydroxylamine is an intermediate product in nitrogen fixation is, however, rejected by Burk & Horner (49, 50). The latter investigators show convincingly with the aid of the manometric method that *Azotobacter* is unable to utilize hydroxylamine as a source of nitrogen. If this compound therefore occurs in culture media of *Azotobacter* it must be considered as a product of a secondary metabolic process. On similar grounds many common nitrogenous compounds, such as urea, ammonia, nitrite, nitrate, hydrazine, asparagine, glycine, and others, do not appear to behave sufficiently like nitrogen in the over-all growth process to indicate that they are specific intermediates in fixation. It was found, however, that *Azotobacter* growing in media containing compounds with amide nitrogen shows a similar sensitivity toward molybdenum stimulation as in the case when free nitrogen is the only nitrogen source. Some amide may therefore well be an intermediate product in nitrogen fixation.

The same authors (51) have studied the occurrence of ammonia in *Azotobacter* cultures and give convincing arguments in favour of the opinion that, contrary to the views of Kostytschew, Winogradsky, and others, this compound results from an ammonification process of primarily-formed cell nitrogen.

A quantitative study regarding the nitrogen distribution in the proteins of four *Azotobacter* species has been made by Greene (52); arginine and lysine were the amino acids present in largest amounts. Approximately 40 per cent of the total nitrogen was found in the

non-basic fraction (simpler amino acids: glycine, alanine, etc.). Indications were obtained that the proteins present were chiefly globulins, glutelins, and albumins.

The aerobic decomposition of complex natural compounds.—Hermann & Neuschul (53) give a detailed account of the hydrolytic abilities of a special strain of *Bacillus mesentericus vulgatus*, to which the name of *Bacillus mesentericus hydrolyticus* was given. Cultures of this organism showed a powerful disintegrating effect on the cell structure of various vegetables, either fresh or boiled. From these cultures very active enzyme preparations were obtained which had the same property and, in addition, caused rapid hydrolysis of starch and sucrose.

The decomposition of alginic acid by some aerobic bacteria is the subject of two publications by Waksman and collaborators (54, 55). Alginic acid, which is widely distributed in marine algae, is a complex carbohydrate belonging to the group of polyuronides; on acid hydrolysis it yields only mannuronic acid. The bacteria hydrolyze pure alginic acid to simpler compounds, still containing several mannuronic acid units, but not to mannuronic acid itself. Active enzyme preparations were obtained which hydrolyze not only alginic acid, but also starch and various other polysaccharides.

Bacterial decomposition of chitin has been investigated by Benton (56) and Bucherer (57); both studies, however, deal chiefly with the very divergent bacterial species involved in the process of natural chitin decomposition.

Horowitz-Wlassowa & Livschitz (58) studied the decomposition of fats by micro-organisms. According to these authors two types of decomposition can be distinguished. The greater part of the bacterial species studied bring about an initial hydrolysis of the fats; a few species like *Bacterium lactis aerogenes* should be able to oxidize the fats directly.

An inquiry into the conditions affecting polysaccharide synthesis from sucrose by bacteria has been made by Cooper & Preston (59); besides the well-known spore-forming bacteria which produce fructosans of the laevan type, various plant pathogens like *Pseudomonas pruni* were shown to perform the same function.

THE ANAEROBIC METABOLISM OF HETEROTROPHIC BACTERIA

Lactic acid fermentation.—The fermentation of glucose by the so-called beer sarcina (*Pediococcus damnosus*) has been studied in

some detail by Mees (60) and was found to yield exclusively *dl*-lactic acid. Dairy scientists remain interested in the formation of acetyl-methylcarbinol and diacetyl in starters, soured cream, butter, etc. Michaelian & Hammer (61) have confirmed previous observations according to which the greater part of these compounds does not originate from lactose, but from citric acid present in the milk. Special additions of citric acid led to considerably increased yields. Ritter & Christen (62) arrive at the same result.

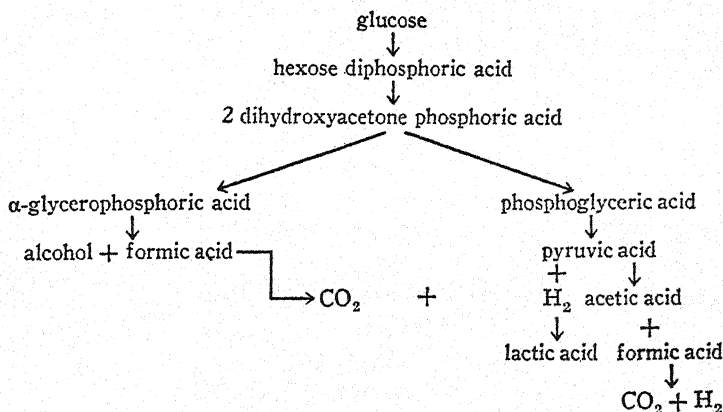
Fermentation by representatives of the colon-aerogenes group.—With a view to the differentiation of the various members of the group renewed attention has been given to the behaviour of these bacteria towards the rarer sugars, poly-alcohols and derivatives. Poe and collaborators (63, 64, 65, 66) investigated trehalose, sorbitol, α -methyl glucoside, and cellobiose; for the various substrates quantitative data regarding the fermentation products are given, showing rather marked differences for the *Escherichia* and the *Aerobacter* subgroups. α -Methyl glucoside was attacked by nearly all *Aerobacter* strains and only by about 15 per cent of the *Escherichia* strains tested.

The fermentation of anhydrides of sugar alcohols: mannitan, mannide, isomannide, and dulcitan has been tested by Dozois *et al.* (67); out of 127 strains none was able to attack these compounds, although mannitol was fermented by all and dulcitol by most strains.

With regard to the mechanism of the glucose fermentation there are several papers of interest. Tasman & Pot (68) were unable to corroborate the results of Stephenson & Stickland from which these authors concluded that separate formic- and glucose hydrogenlyases exist in *B. coli*. On the whole the results obtained pointed in favour of the idea that formic acid is an intermediate product in the formation of hydrogen from glucose by *B. coli*. In a second paper Tasman (69) develops considerations on the basis of a large number of fermentation balances both with living reproductive *B. coli* and with suspensions of "resting" cells of this species. These experiments also lead him to the conclusion that formic acid as a rule is an intermediate product in glucose fermentation.

Virtanen (70) and his collaborator Tikka (71) have discussed the reaction mechanism of fermentation of glucose by *B. coli* in the light of the new reaction schemes of Embden and of Meyerhof for carbohydrate breakdown by yeast and muscle. The results obtained by Tikka in his experiments on the fermentation of dihydroxyacetone phosphoric acid, phosphoglyceric acid, glycerophosphoric acid, and

pyruvic acid led to the following scheme for the fermentation of glucose:



It is, however, difficult to see how this scheme, which differs from earlier ones by its rejection of the "independent" origin of the lactic acid, can account for the high percentages of this acid which are often encountered. Neither can it explain why the molecular ratio of ethyl alcohol and acetic acid formed equals more or less exactly that of carbon dioxide and hydrogen (cf. Tasman).

A special study of the fermentation of ethane diol (1.2) (ethylene glycol) by several members of the colon group has been performed by Groenewegen (72). Acetic acid, alcohol, and acetaldehyde were detected as reaction products and numerous quantitative determinations of these products have been made. Arguments are given in favour of the view that in this fermentation dioxane occurs as an intermediate product.

On the correlation of various biochemical properties amongst representatives of the colon group interesting observations have been made by Schaeffer (73).

Butyl alcohol fermentation.—The unknown factor stimulating the formation of butyl alcohol by certain butyric acid bacteria discovered earlier by Tatum, Peterson & Fred⁴ has been investigated further by the same authors (74). It was shown conclusively that the active factor present in potato extract was *l*-asparagine. In addition it was found that both *l*-aspartic acid and *d*-glutamic acid brought about a stimulative effect equivalent to that of asparagine. Appar-

⁴ Cf. *Ann. Rev. Biochem.*, 4, 602 (1935).

ently certain strains of butyric acid bacteria require a four- or five-carbon dicarboxylic amino acid in order to ferment starch efficiently and vigorously.

Langlykke, Peterson & McCoy (75) give an interesting survey of the neutral volatile products formed by fifty-two different strains of butyric acid bacteria which produce butyl alcohol out of glucose and arabinose. The yields and the mutual proportions of these products (ethyl alcohol, butyl alcohol, acetone, and isopropyl alcohol) show wide variations.

Two papers, one by Blanchard & MacDonald (76), and one by Bernhauer & Kürschner (77), deal with the mechanism of glucose fermentation by *Clostridium acetobutylicum*. The first mentioned authors reject the idea that an aldol condensation of acetaldehyde is responsible for the formation of butyric acid. This insight is founded on the negative results of experiments aiming at bringing about such a condensation with propionic aldehyde added to a sugar-fermenting culture. Instead of undergoing condensation this aldehyde was simply reduced to propyl alcohol. The same product was also formed when propionic acid was added to the medium.

Bernhauer & Kürschner infer from their experiments that, in agreement with current reaction schemes, butyl alcohol is formed out of butyric acid, and acetone out of acetic acid via acetyl acetic acid. Acetaldehyde when added to vigorously fermenting cultures was, for the greater part, converted into ethyl alcohol but led also to increased yields of butyl alcohol. Yet also these authors could obtain no indications for the formation of acetaldol as an intermediate product; they are inclined to consider crotonic acid as such.

Reynolds, Coile & Werkman (78) failed to corroborate the results reported by Weinstein and Rettger⁵ according to whom butyl alcohol bacteria produce normal quantities of acetone but little or no butyl alcohol in Robinson's semi-synthetic medium. In these earlier investigations it had also been found that addition of a prolamine or alcohol-soluble protein was essential in order to ferment glucose in the said medium with normal yields of solvents. Reynolds *et al.*, working with seven different strains, amongst which four had been received from Rettger, as a rule obtained a quite normal fermentation of glucose in Robinson's medium. Occasionally, abnormal fermentations occurred in which the acidity failed to break but no instances of abnormal proportions of acetone and butyl alcohol were met with.

⁵ Cf. *Ann. Rev. Biochem.*, 3, 529 (1934).

Tschekan (79) has determined the influence of acetone and butyl alcohol on the fermentation caused by *Clostridium acetobutylicum*. Acetone in concentrations naturally occurring in this fermentation process does not have any appreciable influence. Butyl alcohol has a strong inhibitory effect, which is already marked at concentrations which are usually attained in the industrial process. A short note by Starr (80) deals with the sluggishness of butyl alcohol fermentation. His experiments tend to show that in sluggish fermentations a definite chemical principle is present which is responsible for the abnormal course of fermentation. Another note, by Underkofler (81), gives data regarding the fermentability of xylose by *Cl. acetobutylicum*. Under favourable conditions a good yield of solvents was obtained out of this sugar.

Finally, mention must be made of a recent paper by van Beynum & Pette (82), in which it is shown that a special type of butyric acid bacteria has been practically overlooked until now. A new species of rather universal distribution in nature is described, to which the name *Cl. tyrobutyricum* is given. This species is characterized by its ability to ferment lactates in addition to glucose and fructose; in contrast to the better known species this organism does not attack the di- and polysaccharides.

Propionic acid fermentation.—Chaix & Fromageot (83) have continued the previously reported observations of Fromageot & Tatum⁶ on the occurrence of an activator for the fermentation of glucose by *Bact. acidi propionici* in press juice from potato or clover. In the first place a high grade of purification of the active principle has been achieved. In the further experiments the complications inherent in the growth process could be avoided since it was found that the activator had a direct favourable influence on the metabolic activity of a preformed bacterial population. These interesting results leave no doubt as to the existence of a definite threshold in the concentration of bacteria necessary to obtain an attack on the glucose. Below this threshold no attack whatever occurs; on passing this critical concentration the amount of substrate converted is proportional to the concentration of bacteria in the medium. The remarkable effect of the addition of the activator is that the threshold disappears and that in all cases, independently of the concentration of bacteria, the same amount of substrate is converted per unit weight of bacteria per hour.

⁶ Cf. *Ann. Rev. Biochem.*, 4, 602 (1935).

In two further notes by Chaix (84, 85) additional information regarding these phenomena is given. Firstly it is shown that different strains of propionic acid bacteria behave differently. One strain shows typical minima of active quantities both for glucose and for lactic acid, independently of the duration of the experiment. With the second strain a threshold is observed in experiments of short duration; on prolonged incubation the normal proportionality between quantities of bacteria and of substrate converted is found. The third strain shows a typical critical concentration in experiments with glucose but not in those with lactic acid. In the second note the important result is reported that the activation in question can be brought about by addition of cystine, cysteine, or reduced glutathione to suspensions with bacterial concentrations below the critical limit.

Anaerobic breakdown of cellulose.—Although our knowledge of the metabolism of the anaerobic cellulose fermenters still remains very imperfect, it may be mentioned here that the bacteriological side of this problem has made rather considerable progress in recent years. Reference may be made to the publications of Khouvine (86), Pochon (87), R. Meyer (88), and V. Meyer (89).

The anaerobic breakdown of nitrogenous compounds.—Stickland (90, 91) has continued his studies on the metabolism of the strict anaerobes. In his first paper he shows that proline is reduced by *Cl. sporogenes*, at the expense of the oxidation of alanine, to δ -amino-*n*-valeric acid. Furthermore, the mechanism of the oxidation of alanine and of the reduction of glycine by the same organism was studied in more detail. It was found that alanine loses four equivalents of hydrogen and yields one molecule each of acetic acid, carbon dioxide, and ammonia. The reaction probably proceeds in two stages of two equivalents each, ammonium pyruvate being the intermediate product. As might be expected the reduction of glycine yields acetic acid and ammonia.

CHEMICAL CONSTITUENTS OF BACTERIA

The investigations on the chemical constituents of bacteria are chiefly concerned with problems of medical interest. Especially the study of the chemical composition of the tubercle bacillus remains to fix the attention of various scientists. Thus, Spielman (92) has purified the tuberculostearic acid first isolated by Anderson *et al.* This acid was found to possess the empirical formula $C_{19}H_{38}O_2$ and to yield on drastic oxidation azelaic acid and methyl-*n*-octyl ketone.

These results point to the constitutional formula of 10-methylstearic acid. Macheboeuf and collaborators (93, 94) have started a reinvestigation of the lipoids present in the tubercle bacillus; the work performed until now deals chiefly with the separation of the lipid fractions. Hecht (95, 96) has demonstrated that, contrary to the results of previous investigators, sterols are present in various bacterial species, including *Mycob. tuberculosis*, *Mycob. phlei*, *B. coli*, and others, also when these bacteria are grown in media which initially do not contain any sterols. Investigations on the relation of micro-organisms to carotenoids and vitamin A have been carried out by Ingraham & Baumann (97) and by Ingraham & Steenbock (98). The latter studied the production of carotenoids by *Mycobacterium phlei*; the presence of a large number of pigments was ascertained, amongst these α -carotene, β -carotene, and kryptoxanthin were identified.

Violaceine, the pigment of *Chromobacterium violaceum*, has been investigated by Wrede & Rothhaas (99) and by Tobie (100). The first authors report the isolation of a crystalline product with the empirical formula $C_{42}H_{85}O_8N_5$ or $C_{50}H_{42}O_8N_6$. Only one of the nitrogen atoms appears to have a basic function. Tobie describes conditions favourable for the production of the pigment.

The distribution of flavins in micro-organisms is the subject of a study by Pett (101, 102). *B. aerogenes* and *B. subtilis* contain small quantities, other species none. It was also established that some bacteria transform the green fluorescent flavin when added to the culture medium into an unknown blue fluorescing substance of still-unknown constitution.

The question whether *Azotobacter chroococcum* can synthesize vitamin D is not fully answered by the experiments of Greaves (103); yet there seems no doubt that ergosterol is produced by this organism growing in a simple synthetic medium.

Preliminary investigations of Bourne & Allen (104) point to the occurrence of vitamin C in bacteria.

FACTORS ESSENTIAL IN THE GROWTH OF BACTERIA

Fildes (105) has extended his researches on the nutritional requirements of *Bac. botulinus*. Together with Knight he had already shown that in a special medium this bacterium, like *Bac. sporogenes*, will develop only after addition of tryptophane and of some unknown principle, the so-called "sporogenes vitamin."

Because Burrows (106) had denied the indispensability of both tryptophane and the said "vitamin" for the growth of *Bac. botulinus*, Fildes has repeated his experiments, which led to a confirmation of his previous results. The conflicting conclusions of Burrows are ascribed to the impurity of the amino acid preparations used by this author. Knight (107) confirms the occurrence in yeast extract of a substance essential for the growth of *Staphylococcus aureus*, as first described by Hughes. This *Staphylococcus* factor proved not to be identical with the "sporogenes vitamin." Very active concentrates were obtained from marmite; the substance is a weak base, which can be distilled at low pressure. The factor proved to be also essential for the proper growth of a strain of *B. anthracis*. A study by Fildes & Richardson (108) deals with the amino acids necessary for the growth of *Cl. sporogenes*. The necessity for the use of very pure preparations is stressed and duly considered. It is shown that the bacterium will grow in a medium containing various salts, "sporogenes vitamin," thioglycollic acid, and a number of amino acids. Of the latter, tryptophane, leucine, phenylalanine, tyrosine, and arginine are indispensable, while histidine, cystine, methionine, and valine are highly important and possibly indispensable. It is pointed out that these results indicate interesting relations on the one hand with the nutritive requirements of animals, on the other hand with the outcome of the experiments of Stickland on the sources of energy available for the bacterium in question.

Pappenheimer (109) has very recently obtained a highly active preparation of the "sporogenes vitamin." The preparation has the properties of an unsaturated hydroxy acid of molecular weight about 200. It is non-identical with auxin, Kögl's biotin, and Williams' pantothenic acid.

Koser & Saunders (110) studied the growth factors present in veal infusion and which are essential for the growth of several "fastidious" bacteria, such as *C. diphtheriae*, *S. dysenteriae* Shiga, and *E. typhi*, in a synthetic medium containing asparagine, cystine, tryptophane, and dextrose as sole organic compounds. The growth factors were removed from the infusion by charcoal and could be liberated, although incompletely, from this absorbent by extraction with hot ethyl alcohol or hot acetone. In a preliminary publication by Koser, Saunders, Finkle & Spoelstra (111) the wide distribution of these factors in animals and in plants is shown; ascorbic acid proved to be unable to act as a substitute for the factors in question.

The already impressive list of publications in which the essential rôle of carbon dioxide for the growth of various bacteria is claimed has been extended by a paper by Gladstone, Fildes & Richardson (112). The carefully conducted experiments of these authors tend to show, indeed, that continuous elimination of the carbon dioxide produced in cultures of nine different bacterial species with widely divergent metabolism inhibits their growth.

OXIDATION-REDUCTION POTENTIALS AND BACTERIAL METABOLISM

Clifton and collaborators (113, 114) have studied growth, oxidation-reduction potentials and ferricyanide reduction in stationary and continuous-flow cultures of *E. coli*, both in a peptone and in a peptone-glucose medium. Although the complicated factors involved do not permit an exact specification of the metabolic activities of the bacteria in the various phases of the experiments, yet the conclusion seems warranted that the concentration of the nutritional compounds and of the bacteria control the metabolic activities of the cells as measured by the rate of reduction of ferricyanide. The oxidation-reduction potential apparently was determined by these metabolic activities.

The oxidation-reduction potential of the bacterial pigment toxo-flavin⁷ has been determined by Stern (115). The normal potential at pH 7.0 was found to be -0.049 V. The slope of the various titration curves is atypical throughout the range from pH 4.0 to pH 8.0. The possibility of a two-step reduction has been discussed.

Yudkin (116) has made a careful study of the oxidation-reduction potentials occurring in suspensions of *B. coli*, *B. alkaligenes*, and *Cl. sporogenes* both in the presence and in the absence of metabolites. He arrives at the conclusion already expressed by Elema *et al.*⁸ that the potentials owe their origin to the presence of electromotively active systems in the medium in contact with the electrode. Such systems are obviously excreted by the cells. Attention is drawn to the fortuity of the potentials observed, which apparently are neither correlated with the nature of the organism nor with changes in the metabolism of the bacterial species. It has to be remarked, however, that in the majority of the experiments the organisms were not placed under conditions permitting normal metabolic activities of the cells.

⁷ Cf. *Ann. Rev. Biochem.*, 4, 608 (1935).

⁸ Cf. *Ann. Rev. Biochem.*, 4, 595 (1935).

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THE MICROBIOLOGICAL LABORATORY
TECHNICAL UNIVERSITY
DELFT, THE NETHERLANDS

SOIL MICROBIOLOGY*

BY SELMAN A. WAKSMAN

*Department of Soil Microbiology,
New Jersey Agricultural Experiment Station,
New Brunswick, New Jersey*

The science of the microbiology of the soil focuses attention upon four distinct and related subjects: (*a*) the abundance and specific nature of the microscopic population of the soil; (*b*) the biochemical activities of the numerous members of this population, some of which possess specific physiological characteristics; (*c*) the rôle of these micro-organisms in the processes known or believed to be of importance in plant nutrition and in soil fertility, and (*d*) the soil as a medium for micro-organisms causing plant and animal diseases, and the relation of the soil population to these organisms. A number of important contributions have appeared during the last four years which bear upon these various phases of the microbiology of the soil; some of these involve new ideas, while others tend to confirm previous results and conclusions, but in a more extended manner and by the use of new methods of approach. The literature of this subject, until 1932, has been reviewed elsewhere (1).

The nature of the soil population.—The methods for studying the soil population can be classified under four headings: (*a*) the plate-culture method; (*b*) soil staining, comprising the direct fixation and staining of a definite soil suspension upon a glass slide, and the contact slide method utilizing slides buried in soil for varying periods of time; (*c*) the direct microscopic examination of the soil; (*d*) the enrichment-culture method. The plate method is still extensively used. The numbers of bacteria in soil, as determined by this method, vary widely, generally between 0.3 and 95 millions, the actinomyces between 0.1 and 36 millions, and the fungi between 8,000 and 1,088,000 per gm. of soil (2); these results confirm previous observations. As with higher plants and animals, the abundance and distribution of micro-organisms is controlled by various ecological factors, comprising climatic (atmospheric), edaphic (soil), and biotic (living forms); these factors do not seem to be sufficient, however, to explain the daily and even more frequent fluctuations of bacterial numbers in soil (3).

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Bacteria were shown (4) to persist in dry soils, rocks, and bricks, an important rôle being ascribed to them in the corrosion and weathering of rocks. Bacteria were also found (5) in great abundance in the deep horizons of undisturbed peat bogs; their activities were correlated with the processes of peat formation.

The method of direct staining of soil, which came to be known as the Conn-Winogradsky method, established the fact that the bacterial population of the soil is far greater than that revealed by the plating procedures (6). Numbers of bacteria, ranging from 1,000 to 4,000 millions per gm., have been found in the case of the Rothamsted field soils; a definite relationship was observed between crop yields and numbers, for a series of soil plots. Manure may contain as many as 37,500 millions of bacteria per gm., the greatest number occurring in stall manure and the smallest in "hot fermented" manure; results obtained by the direct method are generally too high, because dead bacteria are counted as well (7). Considerable attention has been recently directed to the so-called soil-plate method, namely, the Rossi-Cholodny procedure (8). This method offers possibilities not only for the study of the qualitative and quantitative nature of the soil population, but also of certain quantitative changes resulting from soil treatment, including the influence of addition of various plant nutrients (9); an increase in moisture content of the soil results in a rapid change of the natural flora of fungi and actinomyces to one in which bacteria predominate (10). The dominant rôle of bacteria in the decomposition of various organic compounds in non-acid and fertile soils can thus be demonstrated (11); actinomyces develop only after the first stages of decomposition have passed and, therefore, were considered as secondary agents. It came to be recognized (12), however, that the microscopic examination of the soil is adapted for the study of the soil population only when used in connection with the cultural, especially the plate, methods; the two methods can compensate one another. The Rossi-Cholodny technic has also found (13) application in the study of the development of plant disease-producing fungi in the soil.

The direct microscopic examination of the soil, using surface illumination, has previously found limited application. Recently, a method has been developed whereby the soil population is studied in an undisturbed condition (14). It was possible to establish definitely, by means of this method, that the soil harbors a distinct and characteristic flora consisting of protozoa, fungi, and bacteria. The various organisms rapidly respond to treatment, especially the addition of

specific organic substances; the intensity of growth on the surface of the soil differs considerably from that within the soil (15). However, this method, as well, does not supersede the plate and cultural methods; that it serves as a useful supplement is evidenced by the fact that the results obtained fully confirmed those previously obtained by the older methods. The "soil-chamber" method proposed by Cholodny (16) also aids in studying the development of micro-organisms upon glass in contact with the soil.

Extensive use has been made of the enrichment-culture method by the veteran soil microbiologist, S. Winogradsky (17), for the study of two of the most important soil processes, nitrogen-fixation and nitrification. Silica-gel plates, impregnated with different nutrients, are sprinkled with particles of soil; the development of the specific bacteria takes place. The number of *Azotobacter* cells in certain soils in France was found to vary from 0 to 12,000 per gm. The addition of nitrogen compounds to the soil, especially ammonium salts, has a deleterious effect upon the development of this organism. An inverse relation between the numbers of *Azotobacter* and nitrogen dressing of soil was also obtained for the Rothamsted plots (18). The conclusion was reached that *Azotobacter* can develop in the soil only when the multiplication of its antagonists is paralyzed by the lack of available nitrogen, provided of course that such conditions as reaction and presence of available phosphate are favorable for its growth. The use of benzoate as a source of energy for *Azotobacter* is of advantage, since it favors specific development of this organism and discourages accompanying forms. Silica-gel plates have also proved useful in studying nitrifying bacteria; by carefully excluding organic compounds, little difficulty was experienced in obtaining development of the specific organisms (19).

Autotrophic bacteria.—The specific nature of the bacteria concerned in the oxidation of ammonium salts to nitrite and of the latter to nitrate continues to arouse considerable interest, just as it did fifty years ago. Questions are still being raised as to whether the whole process may not be more chemical than biological in nature. It is claimed (20) that the oxidation of ammonium salts, in solution and in soil, can be brought about by sunlight in the presence of certain photosensitizing substances, such as titanium oxide and zinc oxide; this process was referred to as photonitrification. Rossi (21) also suggested that nitrification at the surface of the soil is purely physico-chemical; this process was believed to be of special importance in

tropical countries (22). The evidence submitted to substantiate these ideas is inconclusive and raises doubt concerning the validity of the claims; at best, such reactions account for only a small part of the nitrates formed in nature, as compared with that produced by biological agents. Fraps & Sterges (23) repeated some of these experiments and found that the chemical oxidation of ammonium salts is of little or no practical significance in the soils studied; sunlight actually decreased the rate of bacterial nitrification; however, since direct sunlight is exposed to only a small portion of the soil, its destructive action upon nitrifying bacteria is not of great practical importance.

S. Winogradsky (19) partly abandoned his earlier position that the processes of nitrite and nitrate formation are carried out exclusively by two limited groups of highly specific bacteria. He described recently three groups of organisms capable of oxidizing ammonium salts to nitrite: (a) *Nitrosomonas*, rod-shaped organisms with rounded ends, having their optimum at a definitely alkaline reaction (pH 8.6 to 8.8); (b) *Nitrosocystis*, coccus forms producing zooglea, found commonly in forest soils (24) and having their optimum closer to neutrality (pH 7.4 to 7.8); (c) *Nitrospira*, spiral forms with an optimum pH of 7.0 to 7.2. These organisms were also distinguished by their speed of action, the first being most active and the third least. The nitrate-forming bacteria were also classified in three groups, namely, *Nitrobacter*, *Bactoderma*, and *Nitrocystis*. Boltjes (25) could find no differences between the first and third groups of nitrite formers; he confirmed Winogradsky's earlier results.

In addition to the strictly autotrophic bacteria, it is claimed that various other organisms are capable of producing nitrate. Among these are certain forms, like *Hyphomicrobium*, which probably thrives on the organic impurities in the atmosphere (25), thermophilic bacteria (26), facultative autotrophic bacteria capable of decomposing petroleum and oxidizing ammonium salts directly to nitrate (27), and protozoa. The amounts of ammonia and nitrite oxidized by heterotrophic bacteria are so small (28), however, as compared with the classical autotrophic organisms, that one is inclined to agree with Winogradsky in questioning the significance of those organisms in the process of nitrification in nature. The claim (29) that certain chlorophyll-free protozoa (*Chilomonas*) are capable of existing in nature autotrophically and derive their energy by the oxidation of ammonium salts could not be confirmed (30).

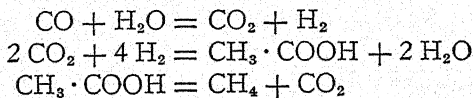
Recent contributions reaffirm the long-standing conclusion that

many of the sulfur bacteria are widely distributed (31). Each soil contains micro-organisms which are active in oxidizing sulfur materials. According to Guittonneau & Keilling (32), elementary sulfur is oxidized in the soil to thiosulfate and polythionates by a variety of heterotrophic bacteria. However, the autotrophic forms do not produce polythionate from either elementary sulfur or thiosulfate; some of the heterotrophic bacteria are able to form polythionates from thiosulfate, the process being favored by an increase in the amount of available organic matter (33). Representatives of the *Eubacteriales* that are able to oxidize thiosulfate include various forms: (a) strict autotrophs (*Th. thioparus* Beij.), (b) facultative autotrophs (*Th. novellus* St.), and (c) heterotrophs (Trautwein's organisms, *Ps. fluorescens*, and others).

The principles underlying the development of the purple and green sulfur bacteria have been established by van Niel; these observations have greatly stimulated study of the organisms (34). They appear to be widely distributed, but their requirements preclude their development as important soil organisms. They are anaërobic and require radiant energy in the form of light which is utilized through the agency of their pigments.

The iron bacteria continue to attract attention; unfortunately, there is frequently insufficient distinction made between the autotrophic bacteria capable of utilizing the energy liberated in the oxidation of the iron, and a variety of organisms which are capable of precipitating iron indirectly; as a result of this confusion, one finds mention not only of iron bacteria, but also of "iron algae," "iron fungi," and even "iron animals" (35).

Carbon monoxide undergoes anaërobic transformations in the presence of hydrogen to form methane (36), the ratios of the initial gas mixtures affecting the course of the reaction. The change occurs in stages and is considered to take place as follows, where high concentrations of hydrogen are present:



The transformation is greatly favored by the presence of various colloidal materials and reducing substances.

Non-symbiotic nitrogen-fixing bacteria.—The life cycle of *Azotobacter* has been receiving considerable attention. It has been shown

(37), for example, that the cells of this organism break up into particles which pass through a bacterial filter; the filterable forms retain their viability and change into coccoid bodies but not into cells of *Azotobacter*; these forms also lack the capacity of fixing nitrogen. *Azotobacter agilis* was found (38) to be quite distinct from *Az. vine-landii*, the former occurring only in canal water and the latter being characteristic of the soil. These two forms are, however, very similar in chemical composition, being high in protein and in hemicellulose and low in lignin-like complexes. *Az. chroococcum* and *Az. beijerinckii*, on the other hand, are low in protein and in hemicelluloses (39). The number of *Azotobacter* in 189 Italian soils was shown (40) to vary from 0 to 21,400 per gm., the abundance of this organism being no proof, however, of extensive nitrogen-fixation in the particular soil. The ability of *Azotobacter* to utilize the products of cellulose decomposition has once more been investigated (41), although no definite answer can be given, as yet, to this important question. The reaction of the soil was at first believed to be the primary factor in controlling the development of *Azotobacter* (pH 6.0); the carbonate-phosphate ratio was found (42) to modify this effect. However, though *Azotobacter* may grow at a pH of less than 6.0, in the presence of fixed nitrogen, nitrogen-fixation takes place only above pH 6.0 (43). The anaërobic nitrogen-fixing bacteria related to *Cl. pastorianum* develop in soil at much more acid reactions (44).

It was believed to have been established (45) that the fixation process proceeds through the ammonia stage. However, Burk & Horner (46) have shown that the ammonia originates from the organic constituents of the cells and that its formation is conditional upon the exhaustion of the energy-supplying material in the medium. Various bacteria which require proteins for their nutrition were found capable (47) of developing in old cultures of *Azotobacter*, while forms which can assimilate amino acids and ammonia grew both in old and in young cultures; this was believed to indicate that soluble nitrogen compounds are produced in young cultures, while in old cultures nitrogen-fixation ceases and only proteins are present. The formation of hydroxylamine as an intermediary product of nitrogen-fixation by *Azotobacter* (48) could not be confirmed (49). The favorable effect of soil extract upon nitrogen-fixation was ascribed (43) to its content of iron in an available form; certain other ions, namely molybdenum or vanadium and calcium or stron-

tium, are also required for nitrogen-fixation, although not for growth of *Azotobacter* in the presence of fixed nitrogen. Soil extract contains organic and inorganic compounds which stimulate the growth of *Azotobacter*; molybdenum cannot take the place of the soil extract, although both favor alike the amount of nitrogen fixed per unit of sugar decomposed (50). Cultures of *Azotobacter* isolated from soils of warm regions seem to be able to fix nitrogen at higher temperatures than cultures from soils of temperate regions (51).

The spirillum described by Beijerinck as a nitrogen-fixing organism was found to be widely distributed in the soil, but was unable to fix nitrogen in pure culture (52). It has frequently been claimed that various soil fungi and actinomyces can fix atmospheric nitrogen, but it has been demonstrated again that they lack this capacity (53).

Azotobacter has been studied extensively as an agent for testing soils for abundance of available phosphorus; for this purpose, the soil-plaque method of Winogradsky has been most commonly used (54). The soil is treated with starch or sucrose, and, in the case of *Azotobacter*-free soils, also inoculated with a heavy suspension of the organism; unfavorable soil conditions, such as acidity, may have to be corrected first. This method has also been used for measuring available potassium in the soil, although certain fungi have been shown (55) to be more effective for this purpose. A test for phosphorus similar to the *Azotobacter*-plaque method has been evolved, making use of the fungus *Cunninghamella blakesleeana* as the testing agent (56). It has also been suggested (57) to determine molybdenum deficiency in soil by the soil-plaque method.

Symbiotic nitrogen-fixing bacteria.—An excellent survey of our knowledge of root-nodule bacteria and their rôle in the fixation of nitrogen by legumes was recently published by Fred and associates (58). The presence of nodule bacteria in the roots of leguminous plants has been found (59) to stimulate the plants to greater photosynthesis, accompanied by active nitrogen-fixation; an increase in carbon dioxide tension in the atmosphere favorably affects the growth of leguminous plants and nodule-production (60), the latter being directly correlated with nitrogen-fixation. The intimate relations existing between nodule bacteria and their hosts in legume symbiosis are largely dependent upon the carbohydrate supply; if the latter is adequate, normal nodule activity results; however, if it becomes deficient, the bacteria either remain dormant in the nodule or attack the tissues of the plant; acidity and insufficient soil moisture may

outweigh the carbohydrate supply in importance (61). The mechanism of nitrogen-fixation by leguminous plants still remains unsettled. The claim that germinating seeds of these plants are able to absorb considerable nitrogen, even when freed from bacteria by sterilization (62), a process stimulated by alkaloids, was not substantiated (63). When nodules are removed from leguminous plants, liberation of ammonia begins to take place, even when the nodules are partially dried; the ammonia was, therefore, considered to be the first product of the nitrogen-fixation process, rather than a product of decomposition of the nodule proteins (64).

The condition of the soil spoken of as "soil fatigue" or "soil sickness," frequently observed in old fields of alfalfa or clover has been ascribed to three causes: (a) the production of a bacteriophage; (b) the existence in soil of different strains of the specific organism possessing different degrees of efficiency; (c) the development of disease-producing organisms, such as nematodes. The first theory has recently received confirmation (65), although it is not universally accepted (66). No filterable form of *Bac. radicola* or ecto-enzyme concerned in nitrogen-fixation was detected during a study of the organism (67). The bacteroids were found to be incapable of reproduction or of giving rise to nodules (68).

Cellulose-decomposing and hemicellulose-decomposing bacteria.—The cellulose-decomposing bacteria have received considerable attention recently. Of particular interest is the existing confusion in regard to the classification of one of the most active groups of these organisms. These filamentous, spirochaete-like, aërobic forms, which occur extensively in soils and in composts, have received at least a dozen different designations, comprising new species, genera, and even families. Originally described by Hutchinson & Clayton as *Spirochaeta cytophaga*, it was named *Cytophaga Hutchinsonii* by Winogradsky. Krzemieniewska (69) found, however, that the two forms are not identical, since the former produces microcysts, while the latter does not; the first was believed to resemble the *Myxococcus* of the myxobacteria, and the name *Myxococcus myxococcoides* was suggested. The germination of the microcysts and their transformation into rods is influenced by the reaction of the medium, temperature, and oxygen tension. The name *Itersonia ferruginea* was suggested (70) for another form belonging to this group. Under certain conditions, the cellulose-decomposing bacteria are adapted to specific nutrition, as shown by the forms found in rice-fields and

iron-rich soils, which require a certain iron concentration in order to produce optimum growth (71).

Considerable attention has also been devoted to the cellulose-decomposing bacteria which grow under reduced oxygen tension; the idea that decomposition of cellulose under anaërobic conditions is brought about by the symbiotic action of several bacteria continues to prevail; the organic acids and gases usually produced in the process are believed to be the products of the secondary organisms acting upon the sugars formed by the true cellulose-decomposing forms (72). A heat-resistant facultative anaërobe, capable of digesting cellulose without gas formation and designated as *Plectridium cellulolyticum*, was found (73) in the stomach of ruminants. Snieszko (74) isolated a thermophilic cellulose-decomposing obligate anaërobe, which grew best at 60° C., and produced acetic acid and ethyl alcohol.

The hemicellulose-decomposing bacteria do not belong as a rule to as limited specific groups, as do the cellulose bacteria. However, various hemicelluloses are attacked by different organisms, which are in some cases quite specific; this is especially true in regard to the polyuronides. The bacteria decomposing the capsular material of *Pneumococcus* belong to the obligate-aërobic, Gram-negative, motile, rod-shaped forms, producing oval spores; they are widely distributed in the soil (75). The bacterium decomposing the specific carbohydrate of *Pneumococcus* Type III was described as *Bac. palustris*; *Flavobact. ferrugineum* attacks the non-type-specific carbohydrate of Type I; *Saccharobact. ovale* attacks the specific carbohydrate of Type II; *S. accuminatum* decomposes the polysaccharide of Type I. The alginic acid-decomposing bacteria are non-spore forming, Gram-negative rods (76); these bacteria produce active enzymes which hydrolyze the specific polyuronides. *Bac. xylanophagus*, an aërobic, spore-forming bacterium also isolated from the soil, was found to decompose xylan energetically (77); several agar-decomposing bacteria have been isolated and described (78).

Other heterotrophic bacteria in the soil.—A number of other aërobic and anaërobic bacteria capable of decomposing a variety of organic substances, such as proteins and their derivatives, various carbohydrates, fats, hydrocarbons, and other substances have been described. The genus *Mycobacterium* is comparatively rare in the soil. It is represented largely by two genera: (a) strongly acid-fast, with little or no acid formation from carbon compounds, of which *Myc. tuberculosis* is a type; (b) weakly acid-fast, with tendency to

produce acid, and showing characteristic cytomorphosis or transformation of long, often-branching rods into cocci, of which *Myc. coeliacum* is a type. They all grow on milk, but do not possess proteolytic or diastatic properties; some are able to decompose acetylene (79).

The genus *Corynebacterium* comprises bacteria which are not acid-fast and do not attack paraffin, but are able to produce acid and are mostly proteolytic and often diastatic. They show a tendency to form branched cells, approaching mycelial development even more characteristic than that of the mycobacteria. A variety of species belonging to this group has been isolated (80) from the soil, including some capable of decomposing cellulose. The forms developing on dextrose-casein agar accounted for 8.65 per cent of all the colonies developing on the plate. These organisms are believed to be identical with various forms described under *Rhizobium*. The occurrence of specific urea-decomposing bacteria in soil has been further studied (81); considerable variation was found in the morphology and physiology of these organisms as well as in the conditions which influence their development (82); they are favored by organic matter and depressed by soil acidity.

Among the pin-point colonies usually developing on the common agar plate, *Bact. globiforme* was believed (83) to be a member of the autochthonous (indigenous) microflora of the soil. Lactose-fermenting thermophilic anaërobes are abundant in the soil, ranging from a few cells to several thousands per gm. (84). Spore-forming bacteria of the *Bac. mycoides* group, functioning in the decomposition of organic nitrogenous compounds in the soil, have received renewed attention (85), after two decades of oblivion.

Actinomyces in soil.—The actinomyces belonging to the *albus* group received monographic treatment by Duché (86); although detailed and comprehensive, this work is limited largely to cultural characterization of the organisms; it lacks sufficient morphological information, which is essential for a complete understanding of this group of organisms. A number of new actinomyces have been described, comprising also thermophilic forms (87). Further emphasis was laid upon the variability of this group of organisms, based upon treatment with ether (88). The ability of actinomyces to utilize carbohydrates is frequently designated as "fermentation" (89), a misnomer, just as it is when applied to a number of aërobic bacteria. The formation of nodules by the alder bush is claimed to be due to

an actinomyces (90); this symbiosis is believed to result in the fixation of nitrogen, a phenomenon which still requires further confirmation.

Considerable emphasis has been laid upon the soil relations of those actinomyces which are capable of producing potato scab. The limiting reactions for growth of these organisms in the soil were found (91) to be pH 5.5 to 6.0 and pH 9.0, with an optimum at pH 7.0 to 7.5; a greater acidity, namely, pH 5.0 or less, either reduced the disease or controlled it completely, although the organism was not eliminated from the soil; at pH 4.78 or less, the potato plant as well became injured (92). The temperature limits for the growth of this organism were 10° and 40° C., with an optimum at 25° to 30° C. An examination of 100 fields in Nebraska revealed the fact that none was free from the scab organisms; cultivation of soil during previous years, frequent growth of potatoes, heavy soil, and high numbers of total actinomyces were found to be associated with high percentage of scab (93).

Fungi in soil.—Isolation and identification of fungi from manure (94) and from different soils throughout the world continues at a rapid rate. The investigation includes studies of fungi from Colorado (95), Bohemian (96) and Indian (97) soils, forest soils (98), salt marshes (99), and sandy soils of the littoral dunes (100). More than 100 species of fungi belonging to forty-four genera have been isolated from Manitoba soils (101). *Saprolegniaceae* were shown to be more widely distributed in the soil than in water, which led to the suggestion that this group be considered as terrestrial in nature (102). As many as twenty-four species of *Fusarium* were isolated from tropical soils; they were found to thrive best in the upper centimeter of soil, except in light-textured soils of the semi-arid region (103). The optimum temperature for the growth of fungi is 20° to 25° C. and the optimum reaction pH 4.0 to 7.0; decomposition of cellulose is favored by a small amount of sugar and retarded by larger concentrations; fungus mycelium is rapidly decomposed by bacteria, the rate of decomposition depending on its nature and age (104).

Soil fungi causing plant diseases.—Various diseases caused by *Fusarium* were correlated (103) with soil conditions; the abundance of the specific organisms in the soil and the type of soil are the determining factors in the amount of disease and in the rate of its spread. Soil type was shown to influence the severity of such diseases

as pea wilt (105), cotton wilt (106), flax sickness (107), root rot of cereals (108), and club root of Cruciferae (109). *Ophiobolus graminis* occurs throughout the upper layers of soil and down to a depth of at least 15 inches (110). The reaction of the soil, moisture content (111), temperature, abundance of organic matter, and treatment are among the most important factors controlling the prevalence of the disease-producing organisms and extent of infection.

Important observations have been made on the influence of the soil microbiological population upon the development of plant and animal pathogens in the soil. The pathogenicity of *Ophiobolus graminis*, the cause of the take-all disease in wheat, was shown to be depressed by the presence of certain specific fungi (112), as well as by the antagonistic effect of the natural soil microflora as a whole (113); *F. culmorum*, *Rhizoctonia* and *Pythium* were depressed by *Trichoderma lignorum* (114). Forest nursery beds which have been steam-sterilized did not give as good growth of the trees as unsterilized beds; however, when they were inoculated with the normal microbial soil population, much better tree growth resulted (115). Certain soil bacteria were shown (116) to be antagonistic to various smuts and other fungi and certain actinomycetes to *Pythium*, the root parasite of sugar cane (117). The controlling effect of applications of organic manures upon the cotton-root fungus was believed (111) to be due to the extensive development of micro-organisms which bring about a condition unfavorable to the pathogen in the soil. Disease-producing bacteria and fungi are also destroyed in the soil by certain protozoa (118). The nutritional conditions of the plant have a direct relation to the soil-born diseases (119). These antagonistic relationships of the various soil micro-organisms to the plant and animal pathogens still represent a field little explored. Various soil protozoa and nematodes are also destroyed by fungi belonging to the *Phycomyces* and to the genus *Pythium* (120). On the other hand, it was found (121) that certain soil fungi (*Cladosporium*, *Alternaria*) may stimulate the action of plant pathogens by injuring the roots of the plants, thus increasing their infectiveness.

Mycorrhiza fungi.—Numerous fungi belonging chiefly to the Basidiomycetes (*Agariceneae*) are capable (122) of forming mycorrhiza, especially with trees; 82 per cent of all the families of dicotyledenous plants form (123) endotrophic mycorrhiza. The relations of mycorrhiza to plant nutrition are still disputed. The endophyte of *Lolium* and of orchids is claimed to be responsible for the

fixation of nitrogen by these plants (124). However, other investigators (125) obtained negative results, suggesting the idea that one is dealing here with a case of parasitism. It was also claimed that mycorrhiza formation renders the plant more resistant to degeneration; defective mycorrhiza development in pine seedlings can be relieved and the growth of the trees stimulated by inoculation of the soil with some material containing the specific fungi; this seems to result in the formation of substances in the soil which stimulate the production of short lateral roots (126). Mycorrhizal association has been found (127) to be an important factor in the metabolism of citrus root cells. The relation of the endophyte in *Calluna vulgaris* has been attributed to the creation of a favorable nutritional environment rather than to an intimate obligate association; similar results have been obtained with *Vaccinium*, the growth of which was stimulated not only by the addition of cultures of the specific endophyte, but also by that of several soil fungi; this led to the conclusion that obligate symbiosis between fungus and host is not established (128). It was suggested that the term "peritrophic mycorrhiza" be used to designate the external fungi living in close relationship to the roots of higher plants, as well as the purely saprophytic fungi living in the rhizosphere (129). The problem of mycorrhiza, as a phase of the broader problem of the numerous relations of soil fungi to the growth and nutrition of higher plants, still awaits detailed fundamental study.

Algae, protozoa, and nematodes in soil.—The humidity of the soil is the most important ecological factor in the distribution of algae; moist soils contain many more species than dry soils; typical algae isolated from lower depths of soil are unable to grow in the darkness; the subterranean algae are looked upon as consumers, bearing no essential relation either to the carbon or nitrogen cycle in the soil (130). Manuring of soil has a decided influence upon the development of specific algae (131).

The method of Cutler for distinguishing between trophic protozoa and cysts was found (132) unsatisfactory, since some of the latter are killed by treatment over night with 2 per cent hydrochloric acid; protozoa were also shown to be more heat tolerant in dry (80° to 85° C.) than in wet soils (50° to 65° C.). The distribution of protozoa in the soil depends largely upon the abundance of humus, as a result of which each soil has its own typical protozoan fauna. Japanese soils were reported (133) to contain 500,000 to 1,000,000

flagellates, 100,000 to 500,000 amebae and 80 to 1,000 ciliates per gm. The fact, however, that protozoa occur in great abundance in the soil and lead there a trophic existence (134) does not necessarily tend to confirm an early hypothesis that these organisms are the controlling agents in soil fertility and are largely responsible for the phenomena brought about by partial sterilization of soil. Without denying the possibility that under certain special conditions, as in water-saturated soils or in the presence of considerable abundance of organic matter, the protozoa may keep bacterial development in check, the evidence so far obtained seems to point to the fact that the results of this relationship are largely beneficial: (a) by feeding on pathogenic bacteria (133); and (b) by keeping important groups of soil bacteria in a state of youth for a longer period of time, thereby stimulating specific bacterial processes (135).

The soil harbors numerous saprophytic (136), as well as parasitic (137) nematodes. The degree of soil infestation with the root-knot nematode can be determined by means of an indicator crop; a susceptible plant, such as the English pea, cowpea, or tomato, is planted in the soil under conditions favorable for growth and infection; after thirty days, sufficient for completion of the life cycle, the plants are removed and the degree of infestation determined by counting the number of galls per plant and percentage of plants infected; the total number of nematodes per given area of soil can thus be calculated (137).

Decomposition of plant and animal residues.—The microbiological investigations of the transformation of organic residues in manures and in soils can be classified into three groups: (a) biological, limited largely to a study of the aërobic and anaërobic population of the manures (138); (b) biochemical, concerned with the mechanism of decomposition of the various chemical constituents of the plant and animal residues by micro-organisms, and with the formation of humus (139); and (c) utilization of the processes of decomposition for the purification of home, farm, and factory wastes (140), for the preparation of composts for mushroom production, etc. Particular emphasis has been laid (141) upon the need for modifying the earlier, purely chemical conception of humus formation and to give greater consideration to the importance of micro-organisms, not only as agents of decomposition, but also as agents of synthesis; the formation of humus is a result of accumulation of the more resistant complexes and their interaction with some of the products of micro-

bial synthesis. Microbial cell substance also undergoes rapid decomposition in soil; a definite relation has been observed between the C:N ratio of the material and the rate of nitrogen liberation (142). Jenny's ideas concerning the inverse variation of the nitrogen and organic matter content of the soil were confirmed (143).

The preservation of nitrogen in soil is largely dependent upon the nature of the organic substances undergoing decomposition. The various chemical constituents do not function alike in this respect; the cellulose and other carbohydrates are decomposed readily, thereby supplying available energy to the micro-organisms, which are able to assimilate the nitrogen and transform it into organic complexes; the lignins bind the nitrogen of both ammonia and protein (144).

Decomposition of certain plant constituents such as cellulose, hemicelluloses, and lignins by micro-organisms has aroused considerable attention. It seems to be definitely established that true cellulose can be readily attacked by a great variety of fungi and bacteria; these comprise the common forms and those growing at higher temperature, namely 65° C. (145). Hemicelluloses are attacked by numerous fungi and bacteria (146). Cellulose is decomposed alike in a natural or in an isolated state; some of the hemicelluloses are more resistant to decomposition after isolation in a free state. This is even more apparent with lignins, probably the most resistant of all the important groups of plant constituents (147). This is due either to the physical or the chemical changes produced in the lignin during the process of its preparation. Lignin is more resistant to decomposition under anaërobic conditions; the partial inhibition of gas formation from cornstalks and packing-house sludge was found (148) to be due not to any toxic effect of the lignin, but to its combination with some of the protein constituents of the residues, thus making conditions unfavorable for bacterial action. Soil bacteria vary in their ability to decompose different amino acids; some of these, such as betaine and valine, are attacked by specific organisms (149).

Mutual relationships between micro-organisms and root systems of higher plants.—The root systems of higher plants serve as a particularly favorable environment for the development of micro-organisms (150). Nodule bacteria appear to benefit not only the host plant but also other plants growing in close association; this was ascribed to the excretion of nitrogenous compounds from the nodules even at an early stage of their development (151); these results were confirmed (152) and were also shown to apply to the

influence of nodule-forming trees upon other trees in forests (153). It has also been reported that soils receiving organic manures, particularly animal manures or composts, produce seed which give higher crop yields than do seeds from plants grown on soil receiving no organic matter; furthermore, the seed is claimed to be more nutritious by reason of increased vitamin content (154). These effects of the organic matter were ascribed to plant stimulants of the nature of vitamins (designated as "phytamins"), elaborated through microbial decomposition of the organic materials; possible relationships between phytamins, vitamins, and plant hormones have been suggested (155). Certain vitamins, at least, are formed in the plants in the absence of influences of micro-organisms (156). Antibiotic effects exerted by soil micro-organisms upon one another may assume particular significance by reason of the fact that some soil-borne plant pathogens appear to be destroyed by products formed by associated saprophytes in the soil environment, as pointed out (112 to 120). A deficiency of manganese in soil, which causes certain specific plant diseases, was found (157) to be brought about by bacterial development.

Influence of environment upon the activities of soil micro-organisms.—A positive correlation was reported between moisture content of soil and the numbers of bacteria, but a less pronounced one between moisture and development of fungi and actinomyces. None of these groups of soil micro-organisms shows any correlation with temperature or with seasonal changes in numbers, except those resulting from variation of moisture. Soil reaction has no effect upon the numbers of bacteria and actinomyces, but is closely related to numbers of fungi. Organic matter content of the soil gives a close correlation with the numbers of bacteria, fungi, and actinomyces, especially the first group of organisms (2).

Common soil bacteria have been demonstrated (158) in the most northern regions (80 to 81°); the general processes commonly associated with soil micro-organisms were shown to have their corresponding representatives in the arctic soils; races (psychrophilic) were found which have adjusted themselves to the lower temperatures, so that nitrification takes place more energetically at 10° C. than at 28° C. Soil bacteria are also capable of adapting themselves to higher temperatures; a definite correlation was observed between the average yearly temperature of the air and of the soil and the optimum temperature for bacterial development (159).

The greatest activity of the micro-organisms in the podsol profile

is controlled by the organic matter relations of the various horizons; it is confined largely to the A₁ horizon (160). In the case of soils in arid regions, two maxima have been observed (161), one near the surface (5 to 20 cm.) and the other near the water-bearing layer (175 to 200 cm.). Forest soils are generally believed to be poor in bacteria and high in fungi; the latter are found largely in the surface organic layers, while the mycorrhiza fungi predominate in the H-horizon. Raw humus soils, because of the high acidity, show lower bacterial numbers and activities; in mull soils, however, where the organic matter is thoroughly mixed with the inorganic portion of the soil, bacterial development is quite extensive (162). Sahara desert soils were found (163) to contain an active microbiological population. Aside from the influence of organic matter and reaction upon the distribution of specific groups of micro-organisms, moisture and temperature are the two most important factors controlling their activities in the soil (164).

Attempts have frequently been made to establish in the soil specific groups of micro-organisms found to be lacking there. Aside, however, from the inoculation of soil with root-nodule bacteria and mycorrhiza fungi, as well as a few other specific organisms, all attempts to secure benefits by inoculation with organic-matter-decomposing or nitrogen-fixing organisms have failed. The inoculation of soil with *Azotobacter* recently received renewed attention, especially in Russia (165); it was assumed that the plant excretes organic substances which provide a source of energy for the bacteria. These results need further confirmation.

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DEPARTMENT OF SOIL MICROBIOLOGY
NEW JERSEY AGRICULTURAL EXPERIMENT STATION
NEW BRUNSWICK, NEW JERSEY

THE BIOCHEMISTRY OF THE FUNGI*

BY N. N. IWANOFF AND E. S. ZWETKOFF

*Department of Plant Biochemistry and Microbiology, University of
Leningrad, Leningrad, U.S.S.R.*

A great part of the investigations on the biochemistry of fungi, carried out during the last two to three years, were devoted to mould fungi. Thus, a considerable amount of data on the intraspecific biochemical variation of the mould fungi has accumulated. A few years ago the investigators wrote¹ that in extending their experiments to *Aspergillus niger*, definite results had been obtained with this fungus. Scientific practice has revealed that within the species *Aspergillus niger* there may be found hundreds, probably even thousands, of strains which, differing but slightly from each other in their morphological characters, show a high degree of variation in physiological and biochemical processes. It has been proved that under the influence of external factors—acidity, temperature, toxins, radiant energy, there may be induced marked changes in the biochemistry of the fungus, and that these changes may be of the nature of a permanent, hereditary modification. It also has been established that the various strains of fungi react in different ways to the introduction of nitrogen and inorganic elements into the culture. In some strains the addition of nitrogen may increase, for instance, the accumulation of citric acid, while in others it produces no effect whatever.

Thus, at the present time our chief attention should be devoted not to the general but to the special biochemistry of the fungi, i.e., to the biochemistry of their individual strains. The differences observed in the various strains allow a deeper study of the biochemical processes to be carried out than would be possible if the strains did not differ from one another. On the other hand, variation makes it possible for us to select the most valuable strains to be utilized for technical purposes (for instance for the manufacture of citric acid).

The biochemical variation of the mould fungi has proceeded farther than their morphological variation. This justifies us in hoping that biochemistry will find more reliable characteristics for the taxo-

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¹ Cf. *Ann. Rev. Biochem.*, 1, 675 (1932); 2, 521 (1933) for a number of papers mentioned in the present text but not listed in the bibliography.

nostic distinction of the various strains of fungi than up to now has been done by morphology.

In addition to *Aspergillus niger*, much work on the biochemistry of other species and genera of the mould fungi has been performed in recent years by Raistrick's school. The data obtained by these investigators on new pigments, acids, and polysaccharides speak on behalf of great polychemism of the mould fungi. Here we have to deal with compounds discovered for the first time in plant cells. Up to the present time only an inconsiderable part of the still fragmentary investigations have been reduced to a system. As soon as this work is accomplished the important rôle of biochemistry in the taxonomy of the fungi will be apparent, and we shall learn much of the biochemical processes taking place in them, which up to now we have been studying in a very one-sided way on *Aspergillus niger* alone.

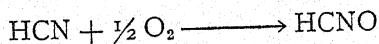
As it is not possible to discuss the whole literature on the biochemistry of the fungi, we have singled out the principal tendencies for presentation in our survey.

NITROGENOUS COMPOUNDS IN FUNGI

As regards the absorption of different forms of nitrogen by fungi, Sakamura, as early as 1930, indicated that in giving ammonium nitrate sometimes the anion and sometimes the cation must be used, depending on the strain of *Aspergillus oryzae* concerned in the experiment. Accordingly the author spoke of "Nitratophilia" and of "Ammoniophilia" in the fungi. Sakamura has now published the continuation of his work (1934) in which he shows that these properties, observed in one and the same fungus, depend on the admixture of salts of heavy metals used in the cultures. The action of iron promotes the absorption of nitrate from ammonium nitrate, and if iron and copper are present simultaneously the utilization of the ammonium ion, i.e., ammoniophilia, is always observed. As the result of these experiments the terms nitratophilia and ammoniophilia have to be discarded.

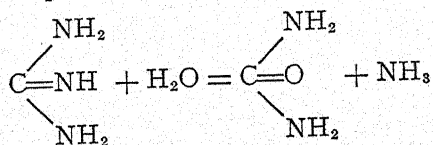
Bach & Desbordes observed in *Aspergillus niger* grown on an acid medium (pH 1.61 to 2.36), the disappearance of nitric acid and its conversion into ammonia. When the fungus is grown on nitrates it may be observed that the nitrate ion penetrates into the cell where it is converted into ammonia and afterwards is disengaged. Having observed the assimilation of nitrogen by *Aspergillus niger* under different conditions of nutrition, Hårdtl indicates that maximum nitro-

gen absorption takes place during the first days of growth, and that deficiency of carbon nutrients in the medium induces extremely rapid and energetic assimilation of nitrogen. Iwanoff & Osnizkaya, who kept *Aspergillus niger* on a sugar solution without nitrogen, observed a regular passage of nitrogen into the surrounding medium. The nitrogen emitted was not further utilized by the fungus, in spite of its complete nitrogen starvation. However, if the fungus starving for nitrogen on the sugar solution was given potassium cyanide as a nitrogen source, the utilization of hydrogen cyanide in some cases reached 100 per cent. This process of nitrogen absorption from hydrogen cyanide is promoted by the addition of sodium thiosulphate to the medium or, according to data as yet unpublished, of powdered sulphur. Sulphur evidently promotes the conversion,



In the presence of sulphur the formation of thiocyanate from potassium cyanide is likewise possible.

Chrzaszcz & Zakomorny (3), in accordance with former data of Iwanoff (1925), have shown that various mould fungi are able to form urea from peptone, and have studied the quantitative influence of sugar, ethyl alcohol, and acetic acid on the accumulation of urea. The authors have confirmed the data of Iwanoff & Awetissowa as to the splitting-up of guanidine by the fungus *Aspergillus niger* by means of a special enzyme, guanidinase, with accumulation of urea according to the equation:



The Polish authors have shown that many mould fungi can accomplish this reaction, and that the addition of a small amount of sugar (0.1 per cent) accelerates the accumulation of urea, while a large amount, on the contrary, retards this process; ethyl alcohol and calcium acetate likewise exert a delaying influence. Chrzaszcz & Zakomorny (4) have come to the conclusion that splitting of arginine does not proceed as usually conceived of, but that guanidine is first formed and this subsequently split into ammonia and urea.

Among the nitrogenous products of fungi, phenylethylamine, found by Keil & Bartmann in the distillate of the fresh fungus *Boletus*

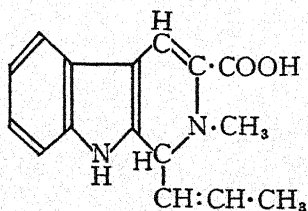
luteus, should be mentioned. In *Boletus luteus* and *B. elegans*, moreover, choline and putrescine have been found. Späth & Zellner have established that the nitrogenous base, marasmine, isolated from *Marasmius scorodoni*, and described by Fröschl & Zellner, is essentially *l*-leucine with which other substances have been admixed. Vorbrodt mentions that during hydrolysis of the protein obtained from *Aspergillus niger* he succeeded in isolating 0.3 per cent of tyrosine.

Bucherer communicates some very interesting data on *Aspergillus niger* and *Penicillium glaucum*, grown on a nitrogen-free medium in which, however, a culture of *Azotobacter* was present to supply the fungus with bound nitrogen. At the same time it was observed that the carbohydrates arabinose, xylose, cellulose, as well as dulcitol, not directly utilized by *Azotobacter*, are converted by the fungi into an absorbable form.

Norman & Peterson have studied the nitrogenous substances in the membranes of fungi. After treating the mycelium of *Aspergillus Fischeri* with alkali they obtained about 20 per cent of a very resistant residue containing about 35 per cent of anhydro hexosamine. This material did not dissolve in cellulose solvents; when treated with weak acids it yielded glucose and hexosamine; this process was accelerated under pressure. Goricca, Peterson & Steenbock obtained a residue containing 2.3 per cent of nitrogen, 62 per cent of which was glucosamine. In connection with the above, mention should be made of the results obtained by Zechmeister & Toth who succeeded in identifying the chitin of the fungus *Boletus* with the chitin contained in the shell of crayfish, by isolating from them the same derivatives—octa-acetyl-chitobiose and undeca-acetyl-chitotriose.

THE ALKALOIDS OF ERGOT

In a series of works Jacobs & Craig have made it clear that lysergic acid is a component of the alkaloids of ergot. The formula of this acid is $C_{16}H_{16}O_2N_2$; it contains an *N*-methyl group and one carboxyl group.



Lysergic acid

A number of authors simultaneously report the discovery of different substances in ergot, giving a special name to each of them. Moir, as early as 1932, discovered in ergot an alkaloid substance, ergobasine, that caused rapid uterine contraction.

According to Stoll, ergobasine has the formula $C_{19}H_{23}O_2N_3$. Moir holds that ergometrine, isolated by Dudley, has the formula $C_{19}H_{23}O_2N_3$, coinciding with that of ergobasine. Jacobs & Craig state that the substance isolated by them from ergot of rye is identical with ergobasine. But ergot contains yet other alkaloids of closely similar molecular weight to that of ergobasine. Thus Kharasch & Legault describe the alkaloid ergotinine, $C_{21}H_{27}O_3N_3$, and Smith & Timmis, ergometrine, $C_{19}H_{23}O_2N_3$, which is an isomer of ergobasine. These newly-discovered alkaloids belong to a group of substances long ago known as alkaloids of ergot. Ergotinine, ergotoxine, ergotamine, ergotaminine are similar as a group but possess different individual characteristics.

CARBOHYDRATES, LIPIDS, ALCOHOLS, AND STEROLS OF THE FUNGI

Mention should be made of the data on starch formation in fungi, obtained by Hida. Among twenty-two species of *Aspergillus* there were nine starch-forming species, in which starch formation took place only in a strongly acid medium. In contradistinction to the data of Chrzaszcz & Tiukow the best formers of starch were at the same time more or less good formers of acids. Kresling was able to distinguish strains of *Aspergillus niger* accumulating much citric acid by the bright blue color of a starch-like substance forming in the tissues of the mycelium when brought into contact with an acid liquid.

Data on the splitting of alginic acid by fungi and bacteria are to be found in the works of Waksman & Allen. These authors have shown that the species of *Penicillium* split both the anhydride of uronic acid and alginic acid, while the species of *Aspergillus* split only alginic acid. Continuing their work on the catabolism of lignin by microorganisms and fungi, Waksman & Smith have established that *Agaricus campestris* splits lignin, but that thereby the number of methoxyl groups very inconsiderably decreases.

Haworth, Raistrick & Stacey have discovered in *Penicillium Charlesii* G. Smith the polysaccharide mannocaralose, which on hydrolysis yields *d*-mannose. According to the data of Raistrick & Smith, *Byssoschlamys fulva* produces a large amount of mannitol, with a yield of 30 per cent from the sugar consumed. Oxford &

Raistrick, during the process of metabolism of glucose, isolated from *Penicillium brevi-compactum* Dierckx and *Penicillium cyclopium* Westling *i*-erythritol. From *Penicillium Charlesii* G. Smith the polysaccharides, polygalactose and polymannose, have been isolated (Clutterbuck, Haworth, Raistrick, Smith & Stacey), which on hydrolysis produce, respectively, galactose and mannose only.

Much work has been done as regards the study of the sterols, especially ergosterol, and of the lipids found in fungi.

Greene & Fred have suggested a method of growing mould fungi on a large scale (see Peterson). This afforded the possibility of studying in detail the lipids of the mould fungi, especially in *Aspergillus sydowii*. In the simple lipids of this fungus Strong & Peterson have found oleinic, linolic, palmitic, stearic, and *n*-tetracosanic acids, as well as ergosterol. The same substances, with the exception of *n*-tetracosanic acid, have been found by Strong & Peterson in the lipids of *Penicillium aurantio-brunneum*. According to the data of these authors the simple lipids contain in percentages: 85.4 of fatty acids (oleinic acid, 40.2; linolic acid, 31.2; palmitic acid, 8.6; stearic acid, 5.3); unsaponifiable substances, 4.5; ergosterol, 1.9; and glycerol, 9.1. The content of lipids in mould fungi grown on synthetic media ranged from 1.1 to 19.9 per cent (average 6 per cent) and on organic media, from 1.5 to 24 per cent. When cultivated on a large scale *Aspergillus sydowii* (Pruess, Eichinger, Peterson) was outstanding by its high content of lipids. The influence of various factors on the formation of lipids and sterols in *Aspergillus Fischeri* has been studied in detail. According to the data of Prill, Wenck & Peterson the accumulation of lipids is promoted by a neutral or slightly alkaline reaction, a high initial concentration of glucose and a low concentration of ammonium nitrate. The amount of sterols increases at a high concentration of glucose, at 1 per cent of ammonium nitrate (or an equivalent content of urea), as well as at a high temperature (37° C) and with a long period of incubation. It has been established that in case of starvation the fungus utilizes a great deal of fat but no unsaponifiable substances. There are indications (Lockwood *et al.*) that if a large amount of fat is to be accumulated in the mycelium of *Penicillium javan.*, the ratio between the surface of the fungus and its volume must be as great as possible, whereby the optimum final pH of the medium is 2.5.

In view of the great practical importance of sterols, especially ergosterols, as initial material for the formation of vitamin D, several

works have been devoted to the determination of the range within which the sterol content varies. Bernhauer & Patzelt have shown that the formation of sterols proceeds, on the whole, parallel to the development of the mycelium. From fifteen different strains of *Aspergillus niger* these authors have isolated several strains containing from 0.23 per cent to 1.16 per cent of sterols. The same authors have found two strains in which the addition of 0.005 per cent of zinc sulphate induced a considerable increase in the weight of the mycelium and in the percentage of sterols, while in two other strains zinc sulphate had a retarding influence. A considerable increase in the accumulation of sterols has been observed in the presence of 2 per cent alcohol. Thus, in cultivating *Aspergillus niger* on 1 per cent sugar, the following results were obtained:

	2 per cent Alcohol	Without Alcohol
Weight of the mycelium in grams.....	0.84	0.15
Amount of sterols in milligrams.....	6.2	1.2
Amount of sterols in percentage.....	0.77	0.78

Up to the present time the rôle of sterols in the organism is unknown. Euler & Klusmann indicate a connection between sterols and carotenoids. In their opinion the products of the breakdown of sugars in green plants, under normal conditions, are converted into carotene and xanthophyll, while in yeasts they lead in some other way to the formation of sterols.

Lukacs & Zellner have found ergosterol, along with other substances, in the fungi *Ganoderma lucidum*, *Hydnum imbricatum*, and *Cantharellus clavatus*. Scheunert and others have investigated the content of vitamin D in mushrooms: 1 gm. of *Boletus edulis* and *Cantharellus cibarius* contains 0.83 rat units of vitamin D; 1 gm. of *Helvella esculenta*, 1.25 units; 1 gm. of *Agaricus psalliota* grown in the dark contains 0.21 of a unit and, when grown under normal conditions, up to 0.63 of a unit. Vitamin D is widespread in fungi and has been found in the powder prepared from edible mushrooms. In various strains of *Penicillium brevi-compactum* Dierckx, Oxford & Raistrick have found ergosteryl palmitate producing on hydrolysis ergosterol and palmitic acid. In some species of *Penicillium* the amount of this ester reaches 0.5 per cent. This indicates that ergosterol and probably also other sterols are found in the plant cell in a bound state.

According to the former data of Tamiya 150 various organic compounds may be used as carbon sources for *Aspergillus oryzae*. At the present time Tamiya, availing himself of the theory of the respiratory coefficient, has succeeded in giving a quantitative expression to the material and energetic metabolism of growth in *Aspergillus oryzae*, and has shown that growth, independently of respiration, is in itself an exothermic reaction. According to Tamiya biological syntheses in a medium consisting of a great number of phases, such as represented by a cell, requires the introduction of a considerable amount of energy. Owing to this circumstance it is possible to explain the variation of the respiratory coefficients in the works of Pfeffer, Rubner, and Terroine, as dependent on the age of the cell, i.e., the time when the organization of the cell changes.

THE GROWTH SUBSTANCES OF THE FUNGI AND VITAMINS

Recently Burgeff has published a work treating the question of avitaminosis in plants, in which the author throws new light on the interrelations of orchids and mycorrhiza fungi.

A characteristic feature of the orchid genus *Vanda* is its strongly reduced embryo which has lost the faculty of independent germination without vitamins or growth substances derived by these orchids from their fungal symbionts. The embryos of these orchids rapidly develop on agar after the mycorrhiza fungus *Rhizoctonia mucoides* has been grown on it. Acetone and alcohol extracts from the mycelium of this fungus, or from beer yeast, produce a similar effect. The active substance secreted by the mycelium of *Rhizoctonia mucoides* is thermostable, not absorbed by filter paper, and does not decompose under the influence of ultraviolet rays or boiling with perhydrol. The author regards this substance as related to the "bios" of Wildiers. It is not specific and may be obtained both from aerial orchids and from the mycelium of *Penicillium*.

The question as to the growth substances of fungi is treated in a series of works by Schopfer. This author has been able to establish that activation of growth in the fungus *Phycomyces Blakesleeanus* may be observed under the influence of the crystalline vitamin B₁, this effect showing itself in an amount of 0.02 μ g. per cc.² The author has ascertained that growth and zygote formation in *Phycomyces* are stimulated by substances isolated from the seedlings of

² 1 μ g. = 1 microgram = 0.001 mg. = 1 γ .

Graminae and from water extracts of the pollen of *Lilium candidum*. Moreover, Schopfer confirms the data of Wassink who has stated that "bios" is an active growth factor for *Phycomyces*. Some *Mucoraceae* develop very well on synthetic nutritive media, requiring no additional growth factors, while other *Mucoraceae* (for instance *Absidia ramosa*, *Phycomyces Blakesleeanus*) fail to develop if growth stimulators are lacking.

Burgeff holds that some of the fungi, *Chaetoclodium* and *Parasitella*, are parasites which have lost the faculty of independently building up vitamins, deriving these from the body of their hosts. Owing to the works of Burgeff and Schopfer the problems of symbiosis and parasitism have been thrown in an entirely new light, and it is from this point of view that the interrelation between these organisms should be studied in the future. Fungi in general have proved useful objects for determining the importance of the individual vitamins in the metabolism of a living cell.

Bünning has investigated the influence of the growth factors, and of vitamins B₁ and B₂, on nitrogen absorption by the fungus *Aspergillus niger*. The said author corroborates the data of Boysen-Jensen (1931, 1932) that the growth substances exert no influence on dry weight increase of the mycelium, but that later, under the influence of growth substances, a rapid degeneration of the mycelium takes place. The growth substances promote the absorption of nitrates and exert a retarding influence on the absorption of ammonia, owing to which the acidity of the medium decreases.

The increased absorption of nitrates, taking place under the influence of growth substances and vitamins, is connected with the facilitated translocation of oxygen and with intensified respiration. In this respect the views held by the author are at variance with those of Sakamura & Yanagihara. The latter consider the growth substances as products of the breakdown of peptone, but think the process to be of a fermentative nature, taking place independently of the oxygen respiration of the fungus.

Kögl & Kostermans report that the growth substance isolated by them from *Rhizopus nigricans* and *Aspergillus niger* is identical with heteroauxin (β -indole-acetic acid). On mineral media, *Aspergillus niger* does not form this growth substance. It appears when peptone, tyrosine, or asparagine have been added to the mineral medium. The author holds that the heteroauxin of micro-organisms is a product of the breakdown of tryptophane.

Thimann & Dolk have studied the conditions under which the growth hormone develops in cultures of *Rhizopus*. Yabuta and his collaborators have shown that, depending on the conditions of cultivation, the mycelium of the fungus *Fusarium heterosporium* produces either a substance accelerating the growth of rice seedlings or one checking growth. The authors have isolated this inhibitory substance, fusarinic acid, $C_{10}H_{13}NO_2$, the yield being 0.5 to 1.0 gm. per 10 liters of culture liquid. Fusarinic acid may be represented as 5-butyl- or 5-*sec*-butyl-picolinic acid.

According to the data of Haenseler, *Rhizoctonia* failed to grow on a nutrient medium on which *Trichoderma* had been grown before. The toxic substance emitted by *Trichoderma* could be destroyed by heating for ten minutes, or by exposure to pure oxygen for five minutes. Paley & Osicheva have observed that the fungus *Penicillium luteo-purpureus* checks growth and acid formation in *Aspergillus niger*. From the nutrient liquid of *P. luteo-purpureus* they have isolated a thermostable substance soluble in ether and chloroform, penicilline, exerting an inhibitory influence on the growth of the fungus *Aspergillus niger* and reducing the yield of citric acid.

Lederer has discovered carotene (provitamin A) in many wild yeasts and fungi. The author has obtained β -carotene and toruline in the crystalline state from *Torula rubra*. In the uredospores of *Puccinia coronifera* α -, β -, and γ -carotene have been found. Moreover β -carotene has been found in *Tremella mesenterica* and in the sporangia of the myxomycete, *Lycogala epidendron*. The latter fungus contains also toruline. Schopfer has isolated β -carotene in the pure condition from *Phycomyces Blakesleeanus*, whereby he noted that a decrease in the asparagine content of the nutrient medium was attended by a decrease in the amount of carotene; the dependence between pigment formation and the quality and quantity of nitrogen in the nutrient medium becomes apparent.

Gorcica, Peterson & Steenbock state that vitamins B_1 and B_4 are contained in the mycelium of *Aspergillus sydowii*.

THE ENZYMES OF FUNGI

Lutz has studied the splitting of cellulose (cotton wool) under the influence of enzymes secreted by *Stereum purpureum*. In the experiments conducted by Lutz the cotton fibers lost their structure, swelled and changed into a paste-like mass in which no oxycelluloses could be found, while cellulose, dextrose, and levulose were present.

According to Lutz the splitting of cellulose passes successively through the following stages: cellulose, hydrocellulose, erythrocellulose, intermediate gums, cellobiose, and glucose.

Menon has investigated the pectinase of apple and potato parasites (*Botrytis cinerea*, *Fusarium*, *Phytophthora*, etc.); it has been proved that in fungi grown on different substrates the activity of the enzymes is not the same; the pectin contained in the substrate stimulates the production of pectinase.

Giesberger, having carried out a comparative study of amylases of different origin, holds that *Aspergillus* contains a mixture of α - and β -amylase.

Kirsh has studied the factors activating lipase in fungi. The optimum pH for the action of lipase is 5.0; inactivation of the enzyme takes place at 60°. The lipase of *Penicillium oxalicum* is activated by calcium chloride; it is rather unspecific, as it effects splitting of various compounds of the nature of esters.

Takahashi & Asai have investigated the fermentative capacity of twenty-four species of *Mucor* under diverse conditions. In one and the same Koji extract the individual species formed different amounts of alcohol. Thus *Mucor Rouxii* and *M. corymbifer* formed 4 per cent of alcohol, and *M. pusillus* only 1 per cent; alcohol formation decreased under free access of air. The formation of alcohol and acids in *Mucor* increased proportionally to the amount of the nitrogen introduced (peptone from 0.05 to 2 per cent). The optimum amount of ammonium sulphate was about 0.3 per cent.

Miwa & Yoshii have studied the conditions under which urease is formed in mould fungi. Having observed the same phenomenon as Iwanoff (1925), namely, that if a fungus grown on a peptone medium is given glucose the amount of urease in the solution considerably increases, the authors explain the phenomenon by increased acidity of the medium. They have stated, moreover, that the formation of urease depends on the life activity of the fungus; with age urease rapidly disappears.

Johnson has separated and studied the properties of proteinase, carboxypolypeptidase, aminopolypeptidase, and dipeptidase obtained from *Aspergillus parasiticus*.

THE PIGMENTS OF FUNGI

This branch of investigation is represented by the works of Rais-trick, Bertrand, Kögl, and their associates.

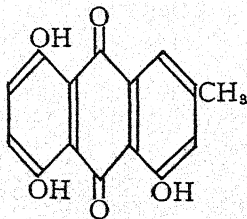
It must be noted that, according to the data of Raistrick & Smith, the fungus *Aspergillus terreus* Thom, when grown on media containing glucose, synthesizes terrein, the empirical formula of which is $C_8H_{10}O_3$. One of the strains of this fungus, under addition of terrein, produces citrinin ($C_{13}H_{14}O_5$), a yellow-colored pigment which formerly could be obtained only as a metabolic product of *Penicillium citrinum* Thom.

According to the data of Oxford, Raistrick & Simonart, the mould fungi *Penicillium griseofulvum* Dierckx, *P. flexuosum* Dale, and *P. Brefeldianum* Dodge produce fulvic acid, a crystalline yellow pigment having the formula $C_{14}H_{12}O_8$.

Gould & Raistrick have isolated a number of pigments from the dry mycelium of *Aspergillus glaucus*: flavoglaucin, $C_{19}H_{23}O_8$, in the form of lemon-yellow needles; auroglaucin, $C_{19}H_{22}O_8$, in the form of golden-orange needles; and rubroglaucin, $C_{16}H_{12}O_5$, in the form of short ruby-red rods. These pigments are evidently specific for the species *Aspergillus glaucus*, as other species of *Aspergillus* do not produce them.

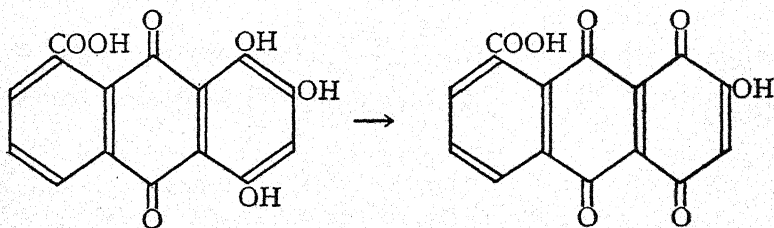
The dry mycelium of the plant pathogen *Helminthosporium gramineum* Rabenhorst, which is responsible for the "leaf-stripe" disease of barley, has been shown to contain two pigments isolated and described by Charles, Raistrick, Robinson & Todd: helminthosporin and hydroxyisohelminthosporin; the former has the formula $C_{15}H_{10}O_5$ and is 2-methyl 4,5,8-trihydroxyanthraquinone; the latter has the formula $C_{15}H_{10}O_6$ and is a derivative of tetrahydroxyanthraquinone.

From the mycelium of *Helminthosporium cynodontis* Marignoni and *Helminthosporium euschlaenae* Zimmermann, Raistrick, Robinson & Todd have isolated a crystalline pigment in bronze-colored plates, cynodontin, which is 1,4,5,8-tetrahydroxy-2-methylanthraquinone or 1-hydroxy-helminthosporin. Cynodontin, $C_{15}H_{10}O_6$, is closely related to helminthosporin, for which the following formula has been established:



Of pigments serving as catalyzers in oxidation processes it is necessary to mention phoenicin, a red pigment discovered by Friedheim in old cultures of *Penicillium phoenicum*; traces of phoenicin may increase the respiration of *Bacillus pyocyaneus* by 200 to 300 per cent.

Bertrand (1897-1902) studied the turning blue of the fungi belonging to the genus *Boletus*, when exposed to air. He isolated from them a red crystalline substance in the form of needles, showing the properties of a phenol and called by him boletol. At the present time (1933) Bertrand has isolated from *Boletus strobilaceus* the substance strobilomycol which with ferric chloride stains green, like pyrocatechol. If acted upon with the oxidative enzyme laccase, the red color of the substance turns black. Kögl & Deijs, following upon Bertrand, have isolated 1 gm. of boletol from 20 kg. of *B. satanas* and *B. luridus*, and have obtained crystals of boletol, $C_{15}H_8O_7$. When exposed to the air, and when oxidized, boletol turns into a quinoid compound, the salts of which stain a pale blue. The color reaction is based upon the transformation of boletol (trihydroxy-anthraquinone carboxylic acid) into hydroxyanthradiquinone carboxylic acid, taking place as follows:



In their second paper Kögl & Deijs report on syntheses both of boletol and isoboletol whereby the synthesized product proved identical with that obtained from the fungus.

ON THE SIGNIFICANCE OF THE INORGANIC ELEMENTS

According to Rippel & Behr potassium influences the synthetic processes in fungi connected with the metabolism of hexose. The authors have proved that the strains of *Aspergillus niger* which accumulate a large amount of oxalic acid contain twice as much potassium in their mycelia and that deficiency of potassium induces the phenomenon of premature old age. Rennerfelt, having introduced a

mixture of salts into the nutrient medium of *Aspergillus niger*, observed that, of the cations, potassium is most rapidly assimilated; magnesium and sodium come next, with calcium and manganese following; the latter are not utilized for the purposes of nutrition.

Several works have been devoted to the question of the stimulation of growth by the salts of heavy metals. Steinberg mentions that it is necessary to discriminate between the action of salt ions as chemical stimulants and as nutrient substances. He points out that the question as to the significance of growth "stimulators" may be solved only by making use of salts entirely free from foreign metals. Having tried the influence of various "stimulators" in the presence of salts thus purified, Steinberg has come to the conclusion that only iron, copper, and zinc are able to stimulate growth, and that the stimulating action of iron must be explained by intensified nutrition of the fungus. According to the author's data "chemical stimulation of growth is possible with a nutrient solution optimum for growth, since an increase in growth through addition cannot under these conditions be ascribed to nutrition, or correction of toxicity of the solution." Lohmann, also, is of the opinion that zinc is a nutrient element of the fungus. Comparing the curves of the action of zinc with those of such unquestionably toxic substances as phenol, the author comes to the conclusion that the curve of the action of zinc coincides with the curves of other nutrient elements. Joshimura noticed that even the pure salts used in cultures usually show admixtures of iron and copper, which in *Aspergillus niger* induces the formation of spherical cells. After purifying the salts by adsorption with charcoal the phenomenon ceases, but may be called forth again by adding the salts of copper, cadmium, nickel, or traces of zinc, but not iron or manganese. According to the author's data the presence of copper and iron is necessary for the growth and formation of conidia.

According to the data of Rabinowitz-Sereni the fungi *Penicillium glaucum*, *Botrytis cinerea*, and *Alternaria tenuis* fail to grow on a nutrient medium from which magnesium is absent; traces of magnesium induce growth, though spores are not formed; magnesium sulphate, raised to 0.04 per cent, increases the dry weight and stimulates spore formation. Butkewitsch has shown that when the strains of *Aspergillus niger* begin to produce low yields of citric acid, the addition of magnesium salts to the solution suffices to increase considerably the accumulation of citric acid. Gudlet, Kirsanowa & Maka-

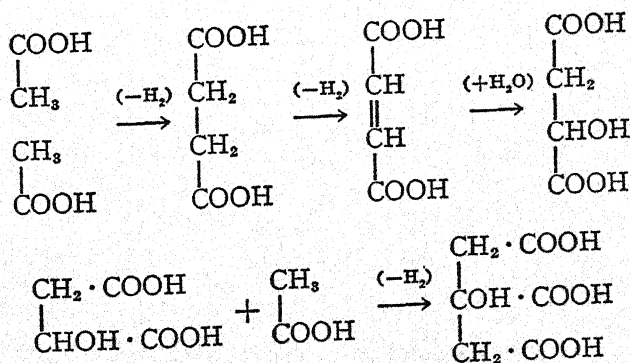
rowa have come to the same conclusion, adding 0.2 gm. of magnesium sulphate to 100 cc. of solution poured over the fungal mycelium. In strong formers of citric acid no increase in the amount of the latter could be observed.

As regards work on the diagnosis of soil-phosphorus content by means of fungi, it is necessary to mention the data of Mehlich, Fred & Truog who availed themselves for this purpose of the development of the fungus *Cunninghamella*. The fungus was grown in Petri dishes on samples of various soils and the authors observed every forty-eight hours that the diameter of the growing mycelium was almost proportional to the amount of phosphorus absorbed from the soil.

Challenger & Higginbottom have published very interesting data on *Penicillium brevicaulis* which, according to these authors, produces a volatile aromatic substance containing arsenic, trimethylarsine.

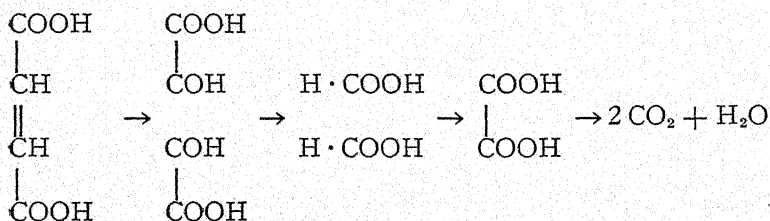
THE ORGANIC ACIDS OF FUNGI

In studying the acids of fungi the conditions required for the formation of citric acid by the mould fungi have received special attention in view of the practical value of this acid. In the last few years all investigators were agreed that the synthesis of citric acid from sugar proceeds through the intermediate phase of acetic aldehyde. The data of Chrzaszcz & Tiukow (1931) and of Bernhauer & Sibenäuger (1931) led to the conclusion that one of the most probable courses was conversion in the following sequence: ethyl alcohol \longrightarrow acetic acid \longrightarrow succinic acid \longrightarrow fumaric acid \longrightarrow malic acid \longrightarrow citric acid, according to the scheme given below:



The starting point for this hypothesis was the discovery of Butkevitch & Fedoroff (1929-1930) that the fungus *Rhizopus* forms succinic acid through dehydrogenation of two molecules of acetic acid. Recently this, it would seem, well-grounded experimental hypothesis has met with objections on the part of Butkevitch, Menzshinskaya & Trofimova (1). The authors declared that the hypothesis as to the formation of citric acid from sugar, through acetic acid, cannot be regarded as well grounded. Without denying the fact that citric acid is produced by the mycelium of *Aspergillus niger* on an acetate solution, the authors hold that the citric acid is formed from the substances of the mycelium, while the addition of acetate only promotes the process. Between the consumption of acetic acid and the accumulation of citric acid there exists no direct connection. Continuing their investigations the authors (2) have shown that under these conditions the chief product of the transformation of acetate is oxalic acid, while citric acid is formed not from acetic acid but from the mycelium, at the expense of substances reducing Fehling's liquid contained in it.

Chrzaszcz & Zakomorny (1) approach the question as to the formation of organic acids in a different way. Adopting the generally recognized scheme for the formation of citric acid through fumaric acid, the authors prove that the calcium salt of fumaric acid is a good carbon source for the species of *Penicillium* and *Aspergillus*, whereby a large amount of formic acid and a smaller amount of oxalic, glyoxalic, citric, and malic acid are accumulated. Transformation proceeds according to the scheme: fumaric acid \longrightarrow glyoxalic acid \longrightarrow formic acid \longrightarrow oxalic acid \longrightarrow carbon dioxide.

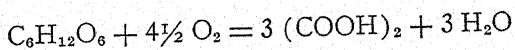


Bernhauer & Slanina (1933) have shown that, if the mycelia of *Aspergillus niger* are given the sodium salt of formic acid, there may be obtained over 40 per cent of oxalic acid. Under the influence of the enzyme formic-dehydrogenase a part of the formic acid is converted

directly into carbon dioxide. On the basis of this reaction the authors have drawn up a scheme for the biochemical transformation of oxalic acid.

Butkewitsch (1934) treats differently the rôle played by the transformation of formic acid salts in the accumulation of oxalic acid by the fungus. In his opinion they are sources of the base which binds oxalic acid, the latter being formed not from formic acid but from the substances of the fungal mycelium. Butkewitsch holds that when oxalic acid is formed from sugar the latter splits, not into two tri-carbon complexes but into three dicarbonic compounds, as from one molecule of sugar three molecules of oxalic acid may be formed. If one adopts the view of Virtanen that glucose splits into two complexes of two and four carbon atoms then the four-carbon compound may continue to split, with the formation of two-carbon substances.

That formation of oxalic acid in this way is possible has been shown by experiments of Tschesnokow, who, having found that the ratio between oxalic acid formed and carbon dioxide emitted is equal to three, holds that the molecule of glucose splits into three molecules of oxalic acid according to the equation:



Bernhauer & Slanina (2), in their further work, have shown the possibility of the fungus converting over 60 per cent of formic acid into oxalic acid, and they draw a parallel between this reaction and the formation of succinic acid from acetic acid. According to the data of these authors high yields of oxalic acid may be obtained also from other acids: from acetic acid, up to 77.6 per cent; from succinic acid, 68 per cent; from fumaric acid, 67.2 per cent; and from glycolic acid, 66.6 per cent. The authors refute Butkewitsch who holds that oxalic acid is formed from the mycelium of the fungus, as, in using formic acid in the experiments, the yields of oxalic acid attained 103 to 160 per cent, calculating from the weight of the mycelium. Bernhauer discards Butkewitsch's hypothesis as to the formation of citric acid from the substances of the mycelium when acetates are used in the experiment. Experiments are mentioned in which the mycelium did not decrease in weight, while the fungus accumulated 0.25 gm. of citric acid at the expense of a decrease in weight of 0.68 gm. of acetic acid. There have been cases where the weight of the citric acid formed considerably exceeded the weight of the mycelium.

But even the established fact that succinic and citric acids are obtained from acetates does not as yet prove that a direct conversion takes place. The possibility is not excluded that the acetic acid is used up as material for the formation of carbohydrates which later give rise to succinic and citric acid.

Chrzaszcz & Zakomorny (5) also decisively object to the view held by Butkewitsch. They have observed the growth of the fungus on a 2.5 per cent solution of formic acid inoculated with spores, and the formation of oxalic acid at the expense of formic acid. They state that the accumulation of oxalic acid takes place as the mycelium grows and is not attended by a decrease in its weight. The authors suggest the following scheme for the transformation of formic acid:



Many investigations have been carried on with the object of ascertaining the optimum conditions for citric acid formation. The statement of Kostytschew & Tschesnokow (1927), formerly advanced, that during the accumulation of acid no nitrogen is absorbed, and that the presence of the latter decreases acid accumulation, has found no confirmation. The question has been investigated by Chrzaszcz & Zakomorny. According to the data of Sotnikov & Paley the addition of magnesium nitrate to the sugar solution considerably increases citric acid formation. A thorough investigation of the question has been carried out by Osnizkaya at the laboratory of N. N. Iwanoff. It has been proved that the effect of added nitrate depends on the strain of *Aspergillus niger* concerned in the experiment. For instance:

<i>Aspergillus niger</i>	Fermentation Solution	Yield of Citric Acid in gm. per Flask
Strain Z	Glucose, 19%	10.28
	Glucose, 19% + 0.3% Mg(NO ₃) ₂	10.00
Strain 33 (from Sotnikov)	Sucrose, 19%	2.26
	Sucrose, 19% + 0.3% Mg(NO ₃) ₂	7.30

From the works of Osnizkaya it follows that the addition, also, of nitrogen from ammonia salts increases the accumulation of citric acid, though the effect produced on the various strains of fungi is not

the same. The addition of nitrogen increases acid formation in fungi which are poor formers of citric acid.

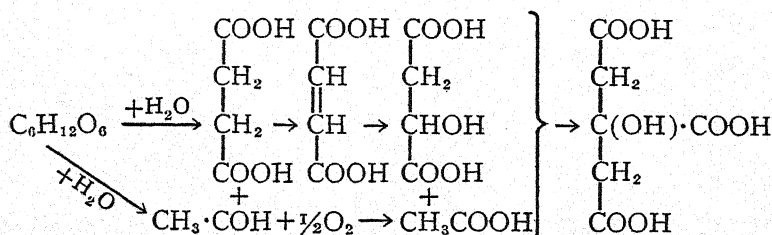
The addition of various salt ions also influences the accumulation of citric acid. Porges (1932) has shown that iron and zinc considerably accelerate this process. Butkewitsch holds that the addition of iron and zinc exerts a favorable influence on the mycelium. Giordani is of the opinion that the salts of iron promote the growth of the mycelium while checking citric acid formation. Chrzaszcz & Peyros corroborate the statement of Bernhauer (1928) that the salts of zinc exert an inhibitory influence on citric acid formation, but at the same time they positively prove that the salts of iron promote the accumulation of citric acid. Recently Wasiliew has shown that the strains of the fungus, in so far as the accumulation of citric acid is concerned, react in a different way to the addition of zinc sulphate. Steinberg holds that iron and zinc are necessary for acid formation, while manganese delays the process.

These contradictions in data depend evidently on the various strains used, the individual strains showing great differences as regards nitrogen and mineral nutrition. The choice of the strain is of great importance for the formation of citric acid (see Chrzaszcz and also Bernhauer). According to the data of Chrzaszcz & Peyros an effective means for increasing the yield of citric acid is partial neutralization with calcium carbonate. The best materials for the formation of citric acid are sucrose and technical glucose. Maltose, molasses, and treacle are less desirable. Paley & Franzusova, by carefully selecting the strains of *Aspergillus niger*, have succeeded in obtaining fair yields of citric acid from molasses. Schwartz & Lang have observed that the addition of arsenic compounds stimulates citric acid formation. The optimum acid-producing regimen for securing maximum yields of citric acid has been studied.

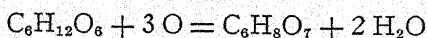
Gudlet, of our laboratory, has established that soon after the process of citric acid formation has begun the oxygen in the solution becomes exhausted, and that a too slow or too rapid supply of air above the mycelium reacts unfavorably on the yield of citric acid. The author states that the weight ratio between the acid accumulated and the carbon dioxide emitted, in many cases, exceeds the value 1.45 admissible from the point of view of the theory of citric acid formation according to the scheme of alcoholic fermentation:



In the course of his work Gudlet has obtained yields of citric acid which, in relation to the sugar consumed, exceed 71 per cent—the maximum yield permitted by this theory. However, as justly remarked by Bernhauer & Slanina (2), the relation between the citric acid formed and carbon dioxide cannot exactly reflect the course of the reactions, as, besides the processes of breakdown, there may take place resynthesis of the sugars. On the other hand, it is possible that pyruvic acid, being transformed by the fungus, undergoes decarboxylation; it is possible that it is directly converted into acetic and formic acid. Taking into consideration the suggestion of Virtanen that, with propionic acid fermentation, the splitting of the molecule of glucose into acetic aldehyde and succinic acid is possible without waste of carbon in the form of carbon dioxide, Gudlet represents the course of the reactions as follows:

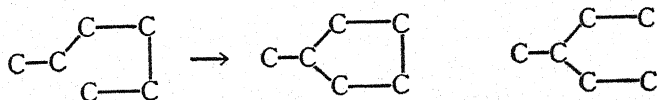


Such a scheme makes it possible to explain the process of citric acid formation without introducing decarboxylation, and thus to reconcile this scheme with the fact that in a series of experiments, conducted by Butkewitsch and other authors, the yield of citric acid, when calculated from the glucose consumed, exceeds 71 per cent, i.e., the extreme figure permitted by the fermentation theory. Recently Butkewitsch & Gaewskaya have carried out an investigation using very careful methods in respect to both the remaining sugar and the citric acid formed. The authors show that the yield of citric acid from the consumed sugar constitutes about 100 per cent. This causes us to return to a scheme long ago advanced by Butkewitsch (1924), the scheme of direct oxidation of glucose according to the equation:



This scheme excludes the formation of citric acid from the prod-

ucts of fermentation, causing it to pass from glucose through intramolecular aldol condensation with formation of a five-membered ring and subsequent oxidation and rupture of the ring, leading to the formation of a branched chain of citric acid:

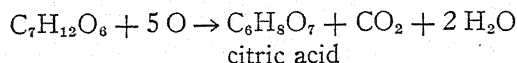
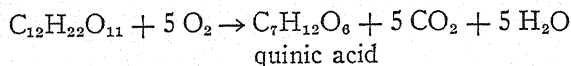


Here the opinion is set forth that between the anaërobic splitting of carbohydrates and the oxidative processes of respiration there exists no genetic connection. Lundsgaard and also Lipmann choose such conditions for their experiments under which only the processes of anaërobic fermentation would be suppressed, while the oxidation processes would proceed normally.

On the other hand, there exist data of Wieland & Sonderhoff (1) that, in the anaërobic fermentation of citric acid with yeast, there are obtained per molecule of citric acid two molecules of acetic acid and two molecules of carbon dioxide, as well as a certain amount of formic acid. Bruce has cultivated *Salmonella aertrycke* on citric acid and has observed that for one molecule of citric acid decomposed there are formed 1.5 molecules of acetic, 0.75 of succinic, and 0.25 of formic acid. Wieland & Sonderhoff (2), in the case of anaërobic dehydrogenation of acetates with yeast, have obtained succinic acid and citric acid.

The total body of fact concerning the formation and decomposition of citric acid by fungi speaks on behalf of its possible genetic connection with succinic, acetic, and other acids. On the other hand, the latest facts published by Butkewitsch & Gaewskaya as to the almost 100 per cent yield of citric acid from the glucose consumed, must cause us to renounce the hypothesis of its production from sugar through the scheme of alcoholic fermentation. The scheme of the splitting of glucose directly into succinic acid and acetic aldehyde, suggested by Virtanen, helps us to draw up a scheme of citric acid formation which excludes the stage of alcoholic fermentation.

Among the theories of citric acid formation there should be mentioned the attempt of Emde to explain the conversion of sucrose through quinic acid into citric acid, according to the scheme:



This theory arose after Fischer & Dangschat had shown the possibility of oxidizing quinic acid by means of periodic acid into citric acid. But according to this theory the yield of citric acid would be maximal at 56 per cent, whereas a number of authors have obtained a much higher percentage of this acid. In the opinion of Chrzaszcz & Peyros this circumstance deprives the hypothesis of any probability.

Among the researches endeavoring to throw light on the formation of citric acid by fungi there remains for mention the investigations of our laboratory. When exposed to the influence of radon both the appearance and the biochemical properties of *Aspergillus niger* changed, giving rise to forms which, instead of citric acid, began to produce gluconic acid (Kresling & Stern). Kardo-Ssyssojewa has shown that degenerative strains of the fungus may be activated by repeated passage through the nutrient medium with sulphuric acid, whereby this acquisition retains its hereditary character.

As regards other acids found in cultures of *Aspergillus niger*, it must be mentioned that Hida, by adding sulphite to the nutrient medium, obtained pyruvic and methylpyruvic acids. The acids are formed from sugar only in the presence of oxygen. Kanel reports that a fungus related to *Rhizopus japonicus*, when grown on media with chalk, accumulates lactic acid at the expense of cane sugar or chalk, the yield being 40 per cent as calculated from the sugar consumed. As the fungus grows, fumaric acid appears in addition to lactic acid.

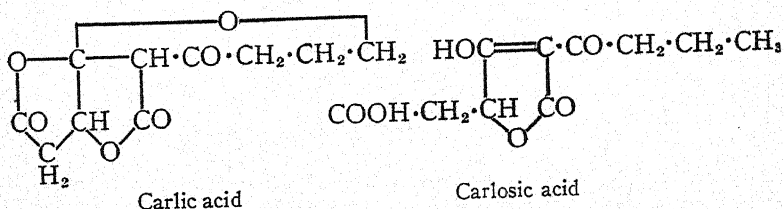
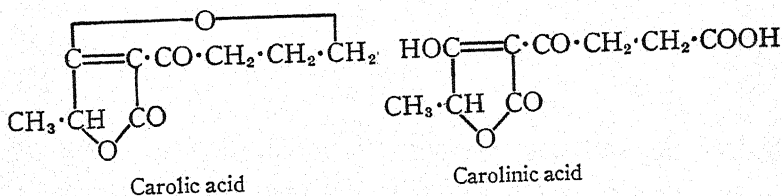
Acids discovered by Raistrick and associates.—Among the most eminent works on the biochemistry of the fungi the investigations of Raistrick and of his numerous collaborators should be mentioned. The authors of the present survey had very many of these papers at their disposal, but only the most important data can be discussed in these pages. For the rest we refer the reader to the *Biochemical Journal* (1933 to 1935) where these important investigations have been published. Of the mould fungi, *Penicillium Charlesii* G. Smith has been studied by Raistrick in the most detailed way. A number of other fungi from the mouldy seeds of corn have also been iso-

lated. These moulds secreted various substances which have given rise to the question whether there is any connection between the substances produced by the fungi and the pellagra of human beings using corn for food. Clutterbuck, Haworth, Raistrick, Smith & Stacey have isolated six new organic acids from *P. Charlesii* G. Smith.

Carolic acid	$C_9H_{10}O_4$
Carolinic acid	$C_9H_{10}O_6$
Carlic acid	$C_{10}H_{10}O_6$
Carlosic acid	$C_{10}H_{12}O_6$
Ramigenic acid	$C_{16}H_{20}O_6$
Verticillic acid	$C_{26}H_{32}O_{11} \cdot H_2O$

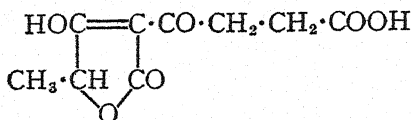
Carolic and carolinic acids are derivatives of γ -methyltetronic acid.

Clutterbuck, Raistrick & Reuter assign the following structural formulae to these acids:

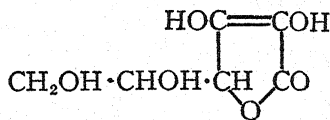


A more detailed investigation of ramigenic and verticillic acids has shown that these two substances are not products of the vital activity of the fungus itself, but arise later through condensation of *l*- γ -methyltetronic acid with acetone used for precipitating polysaccharides. Ramigenic acid has been synthesized from *l*- γ -methyltetronic acid and acetone and has proved to be acetonyl-isopropylidene-*bis*- γ -methyltetronic acid.

There is a strikingly close connection between carolinic acid (I) and the structure of *l*-ascorbic acid (vitamin C) (II).

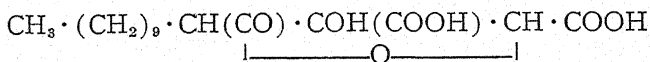


I. Carolinic acid

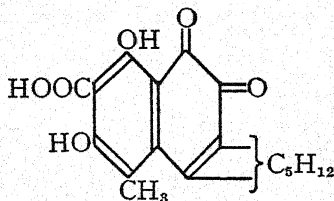


II. Ascorbic acid

A new acid has been discovered by Birkinshaw & Raistrick: *Penicillium minio-luteum*, when grown on a nutrient solution of glucose and mineral salts, produces an acid which is called, after the fungus, minio-luteic acid, with the empirical formula $\text{C}_{16}\text{H}_{26}\text{O}_7$. It has been established that this acid is the γ -lactone of $\alpha\beta$ -dihydroxy- γ -dicarboxy-*n*-tetradecanoic acid, i.e., its structure is as follows:



As regards the products of metabolism of *Penicillium brevicompactum*, Clutterbuck & Raistrick isolated mycophenolic acid, with the empirical formula $\text{C}_{17}\text{H}_{20}\text{O}_6$. The same authors give the structural formula for *normycophenolic acid* $\text{C}_{17}\text{H}_{18}\text{O}_6$:



In this formula only the group, C_5H_{12} , remains undetermined. Raistrick & Simonart have cultivated the fungus *Penicillium griseofulvum* on glucose and have isolated from it gentisic acid which is 2,5-dihydroxybenzoic acid.

Moreover, for the first time, the authors have discovered fumaric acid in this fungus belonging to the genus *Penicillium*.

CONCLUSION

After having reported a whole series of successes achieved in the field of the biochemistry of the fungi we must, however, admit that

the problem of organic acid formation in fungi has not become much clearer than it was when our previous survey was made (1933). A great deal of work will be required before full light has been thrown on the genesis of organic acids in fungi. Many valuable results have been obtained in the search for fungi serving as sources of vitamin D, of the vitamin-B complex, and even provitamin A (carotene). But what strikes the investigator most is the diversity of ways in which the fungi convert glucose into different acids, pigments, and alcohols, as shown by Raistrick's school. Here the prospects which open up in connection with obtaining new organic compounds are indeed unlimited.

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DEPARTMENT OF PLANT BIOCHEMISTRY AND MICROBIOLOGY
UNIVERSITY OF LENINGRAD
LENINGRAD, U.S.S.R.

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